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An anatomical, ultrastructural, autoradiographic,
and cytophotometric study of early embryo,
endosperm, and ovule development in soybean
(*Glycine max* L Merr)

Mark Alan Chamberlin
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**An anatomical, ultrastructural, autoradiographic, and
cytophotometric study of early embryo, endosperm, and ovule
development in soybean (*Glycine max* L. Merr.)**

Chamberlin, Mark Alan, Ph.D.

Iowa State University, 1993

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**300 N. Zeeb Rd.
Ann Arbor, MI 48106**

**An anatomical, ultrastructural, autoradiographic, and cytophotometric study of early
embryo, endosperm, and ovule development in soybean (Glycine max L. Merr.)**

by

Mark Alan Chamberlin

**A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY**

**Department: Botany
Major: Botany (Cytology)**

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For the Major Department /

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For the Graduate College'

**Iowa State University
Ames, Iowa**

1993

DEDICATION

This dissertation is dedicated to Doreen who has been a source of patience, strength, and love through this long and sometimes arduous process of completing a degree, and to my new-born daughter Rebecca who has made finishing a little more difficult, but who has added a little more significance to it all.

Special dedication to all my teachers, past and present, who have piqued my interest in the intricacies of plant anatomy and ultrastructure, Drs. Harry T. Horner, Nels R. Lersten, Lawrence C. Matten, Donald R. Bissing, and Barbara Crandall-Stotler.

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GENERAL INTRODUCTION

The initial impetus for this study was to better understand why interspecific crosses in soybean sometimes resulted in a lack of seed set. Although fertile genotypes were used in these crosses the ovules generally aborted after a short period of development. An obvious incompatibility exists between genotypes in these interspecific crosses, but at what level the incompatibility is expressed is unclear.

A possible source of incompatibility which would result in the early abortion of the ovule is speculated on regarding the Endosperm Balance Number (EBN) Hypothesis. This hypothesis has been implicated as the origin of hybrid failure in interspecific crosses of a number of economically important species (Johnston and Hanneman 1980; Arisumi 1982; Lin 1984; Parrott and Smith 1986). The endosperm is formed in a fertilization event that is separate from that forming the zygote. The two maternal polar nuclei of the central cell fuse with a second sperm nucleus to give rise to the primary endosperm nucleus. The (EBN) Hypothesis suggests that if there is any incompatibility between the sperm and polar nuclei it would result in the aberrant formation of the endosperm or, absence thereof. Any aberrant development of the endosperm would be reflected in the development of the embryo and likely results in a lack of embryo vigor, or in the extreme case, ovule abortion. Such an example exists in Trifolium, in which hybrid failure among some interspecific crosses was attributed to endosperm failure and eventual ovule abortion (Williams 1987).

The importance of the endosperm in nurturing the embryo is well documented in other taxa. In general, the endosperm of angiosperms has been purported to be a rich source of nutrients and/or growth substances for the developing embryo. The endosperm, therefore, is integral to the early development of the embryo. But the ovular tissues also have a significant role in embryo development by housing the embryo sac and supplying the vascular connection with the whole plant.

The ovular tissues are maternal in origin and, therefore, are genetically distinct from the embryo and endosperm. The fact that these three sets of tissues are genetically distinct suggests that their compatibility is essential to the development of the ovule as a whole. Any incompatibility would likely disrupt the highly coordinated interaction of these tissues and result in ovule abortion. But how the embryo, ovular, and endosperm tissues coordinate their actions toward the successful maturation of the embryo and seed is unclear.

The preceding discussion raises some questions about the role of the endosperm, embryo sac, and ovular tissues in the development of the soybean embryo. Indeed little is known about the interdependent development of these tissues which culminates in the formation of the soybean seed. In this dissertation, I have tried to take an integrated approach to understanding the development of the ovule as a whole. In the three papers that follow I have utilized techniques in light and electron microscopy, autoradiography, and cytophotometry to analyze the sequence of events involved in the early development of the normal soybean embryo, endosperm, embryo sac, and ovular tissues. In so doing I expected to answer some questions concerning the roles of these tissues toward the successful maturation of the seed.

My studies were done in a normal compatible line of soybean and the question of incompatibility in interspecific crosses is not addressed specifically. Previously published studies in soybean have looked at specific phenomena or isolated time frames in the development of the ovule. Therefore, a cohesive story on the integrated development of the tissues of the ovule and the sequence of events therein does not exist for soybean. Because of this lack of a cohesive story there is only a fragmented model of ovule development in normal soybean lines. As a preliminary to understanding how incompatibility is manifested within the ovules of incompatible soybean genotypes, I felt it

was necessary to first characterize the processes of development in a normal compatible system.

An Explanation of the Dissertation Organization

This dissertation is composed of three papers. This option was favored because it permits the presentation of work that has been, or will be, submitted to various journals for publication. The three papers are preceded by a general introduction and literature review, and followed by a general summary. References that are cited in the general introduction, literature review, and the general summary follow the general summary.

The first paper is an anatomical and ultrastructural study on various aspects of embryo, endosperm, embryo sac, and ovular tissue development through the first 35 days postfertilization. In the second paper, autoradiographic evidence is correlated with anatomical data to illustrate the pattern of ^{14}C photoassimilate accumulation and flux within the ovule and its incorporation into the embryo. This paper follows the same developmental time frame as the first paper. The third and final paper is a cytophotometric study of the nuclear size and DNA content of the embryo and endosperm from the globular through the late heart-shaped embryo stages.

Literature Review

Endosperm Balance Number hypothesis and polyploidy

Recent attention has been given by plant geneticists and breeders to the hypothesis of Endosperm Balance Number (EBN). This hypothesis states that a chromosomal locus or a series of loci in the nuclear genomes of the sperm and the polar nuclei determine their compatibility, fusion, and subsequent development of the endosperm. The EBN hypothesis

was proposed by Johnston et al. (1980) to explain abnormal endosperm development in interploidy-intraspecific and interspecific crosses. For successful development, the endosperm must have a ratio of two sets of EBNs from the female parent (one set per polar nucleus) to one from the male parent (Parrott and Smith 1986). For compatibility and successful development of the endosperm and eventual maturation of ovule or seed, a balanced ratio must therefore exist between the number of these EBN loci in the chromosomal complement of sperm and polar nuclei.

Interspecific crosses made in Solanum (Johnston and Hanneman 1980; Ehlenfeldt and Hanneman 1988), Impatiens (Arisumi 1982), and Zea (Lin 1984) have shown that incompatibility between certain genotypes may lead to fertilization, but seed formation was lacking. All these authors proposed that different genotypes within the same species have unique EBNs. Crossing of these incompatible genotypes disrupted the EBN ratio of the endosperm and caused early abortion of the embryo. In Trifolium, for example, certain interspecific crosses resulted in endosperm failure and subsequent ovule abortion (White and Williams 1976; Williams and White 1976). Indeed, evidence for the existence of EBN was reported in Trifolium and has been suggested as a mechanism of reproductive isolation between species of this taxon (Parrott and Smith 1986). Endosperm failure in Lilium hybrids was shown to be due to an accumulation of mitotic abnormalities that result in reduced mitotic activity (Brock 1954). This reduced activity culminated in the degeneration of the endosperm and failure of the embryo.

The embryo is initiated by the fusion of a second sperm with the egg, which are sister cells of those forming the endosperm. The embryo, therefore, inherits the same compatibility factors (EBNs) as does the endosperm. But the embryo has only two sets of EBNs (one set from the sperm and one set from the egg) whereas the endosperm has three sets. Because the development of the embryo and that of the endosperm are closely related,

their compatibility is essential to the successful development of the embryo. It seems likely that EBN factors influence the compatibility of embryo and endosperm and that a fixed ratio of EBNs between these tissues is necessary for compatibility similar to that of sperm and polar nuclei in the formation of endosperm.

If a set ratio of EBN loci between endosperm and embryo is important, it is likely that stable ploidy levels also are necessary to maintain compatibility between these tissues. But polyploidy is widespread in reproductive tissues and organs of angiosperms (D'Amato 1984), especially in seed tissues in which numerous cases of polyploidy have been documented in antipodals (Hasitschka-Jenschke 1959), embryo (Bryans and Smith 1985), and endosperm (Chopra and Sachar 1963).

The endosperm originates by fusion of the two haploid polar nuclei and the sperm nucleus and, therefore, is initially triploid in most angiosperm genera. As the endosperm develops, its nuclei may become polyploid due to mitotic inhibition at prophase or metaphase (Vijayaraghavan and Prabhakar 1984). This inhibition results in either endoreduplication or polyteny. An extreme example of endoreduplication exists in the uninucleate chalazal haustorium of Arum maculatum (Erbrich 1965). This nucleus reaches a maximum ploidy level of $24,576n$, which corresponds to 13 endoreduplications. Indeed the chalazal haustoria, as well as the endosperm proper of a number of taxa, are purported to have high ploidy levels resulting from endoreduplication as reviewed by D'Amato (1984). Reports on polyploidy in the endosperm resulting from polyteny are uncommon, but these giant chromosomes have been noted in Zea mays (Duncan and Ross 1950) and Allium ursinum (Turala 1966). In those taxa that have endospermic haustoria, the common trend is that the haustorium has a higher level of polyploidy than the endosperm proper (D'Amato 1984).

In the embryo, differential polyploidization seems to be related to differentiation of its tissues. Differential amplification of nuclear DNA was observed between the cotyledons and embryo axes of Brassica napus (Silcock et al. 1990) and Phaseolus vulgaris (Johnson and Sussex 1990). In various species of Phaseolus, the suspensor cells have consistently higher ploidy levels than those of the embryo proper (Nagl 1974). This is due to their large polytene chromosomes. Even within the suspensor itself, the cells can be of different ploidy levels. A gradient of increasing polyploidy is exhibited basipetally within the suspensor of Eruca sativa (Corsi et al. 1973). A similar situation exists in cotyledons of the legumes Pisum arvense (Smith 1971), Vicia faba (Millerd and Whitfield 1973), and Phaseolus vulgaris (Smith 1974). The epidermal layer is composed of diploid cells, but the ploidy level of the cotyledonary cells increases centripetally to 64C in the inner storage cells.

It is speculated that polyploidization has a dosage effect that may increase transcriptional and translational potential of the cell without committing energy resources to the processes associated with new cell formation (Clutter et al. 1974). This would be an obvious benefit to tissues which are rapidly growing and have limited resources. A direct correlation between polyploidy and RNA/protein synthesis has been noted in the cotyledons of Pisum sativum (Scharpe and Van Parijs 1973) and Vicia faba (Millerd and Whitfield 1973), and in the suspensor of Phaseolus coccineus (Clutter et al. 1974). Elevated levels of transcription and translation, as a consequence of DNA amplification, also have been reported in the endosperm of maize (Kowles and Phillips 1988), and of wheat (Chojecki et al. 1986).

Whether polyploidy in the endosperm and embryo is the agent of differentiation or only a result of this process is unclear. It does seem clear that polyploidy has a physiological significance that affords functional specialization to the tissues in which it

occurs. Examples of this would be the endospermic haustoria and suspensor and their purported role in nutrient absorption. Cotyledons that serve as nutrient storage organs are another example.

Embryo, endosperm, and ovule anatomy, and embryo nutrition

Double fertilization may be a key element in the evolutionary success of the angiosperms. The double fertilization event insures that nutrients are not allocated to the ovule until there is a successful fertilization of the egg and polar nuclei to form the embryo and endosperm, respectively. Unlike the gymnosperms which form a fleshy nutritive gametophytic tissue prior to fertilization, the angiosperms waste no resources in the formation of a nutritive tissue (endosperm) until fertilization is guaranteed. Indeed, Westoby and Rice (1982) argue that the investment of nutrients to the endosperm is deferred "so that the mother can direct her limited resources to a better subset of offspring genotypes." Those offspring which are perceived to be unfit would be aborted by selectively closing off their access to maternal nutrients. Concerning the allocation of nutrients, "the mother achieves this by responding to differences in the vigor of early growth among offspring genotypes." Further checks on the allocation of reservoirs to the ovule are contingent upon the continued development of the embryo and endosperm, and differentiation of the ovular tissues. This type of feedback communication between the embryo and maternal tissues would insure the successful maturation of the embryo without wasting reserves. Some of the salient features of the ovule which insure the successful development of the embryo will be discussed later.

The endosperm seems to be of central importance in the success of the young embryo. Of all the tissues of the ovule, it has received the most attention in the literature concerning the growth and morphogenesis of the embryo. Historically, the endosperm has

been known to contain "special properties" important to the development of the embryo. Well before it was known what these properties were, researchers were using the liquid endosperm of Cocos nucifera (coconut milk) in the culture of embryos of various taxa in growth and morphogenesis experiments (Van Overbeek et al. 1941, 1942; Norstog 1956). It is known, in the case of coconut milk, that the endosperm contains protein granules, oil bodies, and growth hormones (Vijayaraghavan and Prabhakar 1984). The liquid endosperm of maize and Aesculus woerlitzensis also have been used in tissue culture experiments for their abundance of hormones and growth substances (Raghavan 1986). These growth substances likely have a regulatory role in the growth and differentiation of the embryo.

In taxa which have persistent endosperms, the nutritional benefit to the embryo is obvious. These taxa have endospermic seeds and their endosperm cells are generally rich in starch, lipids, and proteins. The cereal grains are examples of this group. In Ceratonia, the endosperm commonly contains large quantities of storage polysaccharides, usually galactomannans, in the form of heavily thickened cell walls (Bailey 1971). Galactomannans also occur in abundance in the endosperm of a number of Palmae, Leguminosae, Rubiaceae, and Convolvulaceae, presumably in the cell walls (Jacobsen 1984). These walls offer rigidity and protection to the embryo, and then upon hydrolysis, provide nutrients to the embryo at germination.

In taxa that do not have a persistent endosperm, the importance of the endosperm in the nutrition of the embryo is unclear. In most cases the embryo grows rapidly and the endosperm is nearly obliterated before it is fully developed, as in Capsella (Schulz and Jensen 1969) and soybean (present study). Because the endosperm is still developing, incoming assimilates are likely invested in the growth of the endosperm and not in the accumulation of storage reserves. Yeung and Clutter (1978) suggested that the endosperm has to attain a certain developmental maturity before it can store nutrients. Even though

the endosperm cells are "immature," they may be a limited source of nutrients for the embryo. In sunflower (Newcomb 1973) and soybean (present study) a clear zone devoid of endosperm cells forms around the embryo. Remnants of endosperm cells occupy this zone suggesting that the cells break down enzymatically in front of the encroaching embryo. Although the endosperm cells do not have storage products, they may be a source of cell wall polysaccharides, amino acids, and nucleosides for the embryo. During late embryogeny in some genera, the remaining endosperm layers may accumulate reserves for later embryo development. At the late heart-shaped embryo stage in Lactuca sativa, only the outer two layers of cellular endosperm persist, but their cells become engorged with protein bodies and spherosomes that are utilized at germination (Jones 1974).

The presence of wall ingrowths from the central cell wall may allow the endosperm to attract metabolites from the ovular tissues into the embryo sac. Mitochondria and dictyosomes have been observed adjacent to these wall embayments lined by plasmalemma. The structure of this wall-membrane apparatus is similar to transfer cells which are purported to be involved in short-distance translocation of metabolites by enhancing the surface-to-volume ratio of cells (Pate and Gunning 1972). These central cell wall ingrowths have been noted in Pisum (Marinos 1970), cotton (Schulz and Jensen 1977), alfalfa (Sangduen et al. 1983), Vigna sinensis (Hu et al. 1983), and soybean (Folsom and Cass 1986). The wall ingrowths can become massive and surround the base of the embryo as in Euphorbia helioscopia (Gori 1977), Pisum (Marinos 1970), and soybean (Tilton et al. 1984). Brentwood and Cronshaw (1978) established that ATPase activity was greatly elevated in Pisum phloem transfer cells, suggesting an active transport function to these cells. Although the wall ingrowths of the central cell have not been tested for enzymatic activity, they likely function in the transport of assimilates, as well.

Another manner in which the endosperm may extract nutrients from the ovular tissues is by the formation of endospermic haustoria. Regions of the endosperm may undergo differential growth and expansion, and the resultant tissue becomes haustorial-like to invade the outer tissues of the ovule. In most cases, as exemplified in some legumes, endosperm cellularization in the chalazal region of the embryo sac does not occur or is greatly delayed. This results in the formation of a coenocytic appendage that can take on various shapes and sizes (finger-like to bulbous) as it invades the chalazal integumentary tissue (Johri and Garg 1959). In the taxa in which they occur, a chalazal haustorium is the predominate type among angiosperm families (Miksell 1990). Less frequent micropylar haustoria exist in the Ericaceae, Verbenaceae, Scrophulariaceae, and Bignoniaceae. These haustorial appendages can become long, the chalazal haustorium of Cucurbita ficifolia can reach 12 mm in length (Chopra and Agarwal 1958). They also may become highly aggressive and invade tissues exterior to the ovule. The micropylar haustorium of Melampyrum lineare extends to the funiculus (Arekal 1963), while the highly digitate haustorium of Iodina rhombifolia penetrates the fleshy pericarp of the ovary wall (Bhatnagar and Sabharwal 1966). Endospermic haustoria have been observed in a number of genera in 64 angiosperm families (Mikesell 1990). Their presence in such a vast array of families suggests that they are a highly conserved morphological trait which has functional importance. They likely function in the absorption of metabolites from the tissues they invade and the polar transport of these metabolites to the embryo sac and embryo. The presence of highly polyploid nuclei in the haustoria of a number of taxa (D'Amato 1984) suggests an active absorption mechanism.

The liquid endosperm of Phaseolus vulgaris maintains a high osmotic pressure in the central cell by presumably concentrating osmotically active substances (Smith 1973). A high osmotic environment may be essential for the normal development of the embryo. Folsom

and Cass (1992) suggested that the hypertonic osmolarity of the central cell provides an environment around the embryo which stimulates metabolite uptake by the embryo. It also has been suggested that this same environment may inhibit precocious germination (Norstog 1967). Therefore, among its many roles in the absorption and accumulation of nutrients, the endosperm/central cell seems to have an osmo-regulatory function important to early embryo development.

The majority of angiosperm genera demonstrate the nuclear-type of endosperm development. In this "type" the endosperm goes through a period of free-nuclear divisions and cell wall formation (cytokinesis) is deferred to a later time. A number of conflicting reports have arisen as to the actual pattern of wall formation. The most notable of these conflicts occurs in wheat (Mares et al. 1975, 1977; Morrison and O'Brien 1976; Fineran 1982; Van Lammeren 1988). The controversy on the pattern of wall formation in wheat is as follows. Anticlinal walls arise as ingrowths of the central cell wall and their tips grow in the presence (Mares et al. 1975, 1977), or absence (Morrison and O'Brien 1976), of closely associated microtubules. Alternatively, Fineran et al. (1982), and Van Lammeren (1988) indicated that these first anticlinal walls do not arise from the central cell wall, but originate through phragmoplast and cell plate formation and these then eventually fuse with the central cell wall. In all cases, continued wall growth subsequently forms cylinders ("alveoli") that are open centripetally. The formation of periclinal walls within the cylinders occurs when the anticlinal walls branch and the branches subsequently fuse (Morrison and O'Brien 1976). Alternatively, periclinal walls are formed by phragmoplast and cell plate formation following division of the nucleus at the base of the cylinder (Mares et al. 1975, 1977; Fineran et al. 1982; Van Lammeren 1988). These processes form the first layer of endosperm cells along the periphery of the central cell. According to Mares et al. (1975, 1977), Fineran et al. (1982), and Van Lammeren (1988), further cellularization of the

endosperm is by continued extension of the cylinders and partitioning of the cylinders with periclinally oriented cell plates. While Morrison and O'Brien (1976) presume that once the first layer of cellular endosperm is laid down, continued cellularization of the endosperm is by normal cell division, thus, forming an endosperm meristem.

Endosperm cellularization in other taxa follows one or a combination of the above processes. In Stellaria media (Newcomb and Fowke 1973), Helianthus annuus (Newcomb 1973), and Quercus gambelii (Singh and Mogensen 1976), anticlinal walls grow out from the central cell wall in the absence of microtubules. Although this same pattern of wall growth occurs in the first four endosperm layers of Haemanthus katherinae, the fifth and subsequent layers have microtubules perpendicularly oriented to the growing tip of the wall (Newcomb 1978). Tip growth of anticlinal walls is assisted by a similar array of microtubules in cotton (Jensen et al. 1977) and soybean (Dute and Peterson 1992). Formation of periclinal walls may occur by branching in Stellaria media (Newcomb and Fowke 1973) and Iberis amara (Prabhakar 1979), or by phragmoplast and cell plate formation in Helianthus annuus (Newcomb 1973).

These previously discussed studies have elucidated the pattern of wall formation utilizing light and/or TEM. Thus, three-dimensional reconstruction of the pattern of endosperm cellularization was limited to interpretation of two-dimensional micrographs. It is unlikely that the conflict over endosperm wall formation will be settled until unequivocal evidence is produced. High voltage TEM, SEM, and/or confocal microscopy may help to resolve this problem.

In most angiosperm species the egg cell demonstrates a common organization, characterized by a distinct polarity of its cytoplasm (Willemse and Van Went 1984). The chalazal one-third to one-half is filled with cytoplasm containing the egg nucleus and the majority of the organelles. The micropylar one-half to two-thirds of the egg cell is nearly

filled by a single large vacuole that displaces a thin layer of cytoplasm to the periphery of the cell. In Gossypium hirsutum, the organelles of the fertilized egg relocate and become clustered around the apically oriented nucleus (Jensen 1968). This change accentuates the polarity already present in the unfertilized egg. A further expression of polarity is the general lack of a wall over the chalazal end of the unfertilized egg in the majority of genera studied. In most species a cell wall is confined to the micropylar portion, while the chalazal portion is bound by only the plasmalemma. Exceptions do exist in Capsella (Schulz and Jensen 1968), Plumbago (Cass and Karas 1974), and Papaver (Olson and Cass 1981), in that these taxa have a discontinuous chalazal wall. Following fertilization a wall is laid down at the chalazal end of the zygote within two days in Rhododendron (Williams et al. 1984) and Nicotiana (Mogensen and Suthar 1979). According to Raghavan (1986), the formation of a wall "essentially insulates the newly formed zygote from the influence of neighboring cells of a different genotype and probably has some significance in the subsequent induction of the sporophyte development phase."

In general, the first division of the angiosperm zygote is transverse giving rise to a small terminal cell (chalazal) and a large basal (micropylar) cell. The cytoplasmic polarity of the zygote seems to be carried over into the two-celled proembryo. In Capsella (Schulz and Jensen 1968), Quercus (Singh and Mogensen 1975), and Arabidopsis (Mansfield and Briarty 1991) the terminal cell has a dense cytoplasm rich in organelles, while the basal cell has fewer organelles. In most cases the terminal cell produces the organogenetic part of the embryo, and the basal cell the suspensor. Thus, the polarity displayed by the zygote profoundly affects the expression and fate of its daughter cells.

As well as cytological differences earmarking the fate of the cells of the two-celled embryo, their fates may be expressed very early in development as morphological and functional differences. In Stellaria media the basal cell of the three-celled embryo forms

transfer cell-like wall ingrowths from its basal wall (Newcomb and Fowke 1974). Similar ingrowths are noted in the basal cell of the soybean proembryo at a very early stage (Dute et al. 1989; present study). Wall ingrowths are observed in the micropylar end of the zygote of Capsella (Schulz and Jensen 1968), denoting a functional polarity even within this cell. In this latter case, it is suggested that the ingrowths aid in the absorption of breakdown products from the degenerating synergids.

The polarity observed in the two-celled embryo is manifested morphologically by the differentiation of the embryo proper and suspensor. Indeed the embryo proper and suspensor often mirror the cytological differences seen in the two-celled embryo. Generally, the cells of the embryo proper are densely cytoplasmic and have numerous organelles, whereas the cells of the suspensor are highly vacuolate with a reduced number of organelles.

The suspensor is a transient organ which comes in a variety of shapes and sizes as has been observed in the Leguminosae (Lersten 1983). In some taxa the suspensor is highly reduced (Euphorbia and Ruta), or it may be lacking as in Lycopsis, Tilia, and Viola (Natesh and Rau 1984). In those taxa which have prominent suspensors, it is now generally presumed to have a central role in the absorption of nutrients from the central cell and their translocation to the embryo proper. This function is suggested by the presence of wall ingrowths in the suspensor cells of a number of diverse species, Phaseolus coccineus (Yeung and Clutter 1978), Brassica napus (Tykarska 1979), Vigna sinensis (Hu et al. 1983), and soybean (present study). The central importance of the suspensor to embryo growth and differentiation has been shown *in vitro*. In culture, the growth of embryos of Eruca (Corsi 1972), Phaseolus coccineus (Nagl 1974), and P. vulgaris (Smith 1973) are greatly improved by the presence of the suspensor. Gibberellins (Alpi et al. 1979) and cytokinins (Lorenzi et al. 1978) have been shown to have much higher activity in suspensors than in

the embryo proper of Phaseolus coccineus. This suggests that in addition to its role in the absorption of metabolites, the suspensor is a source of growth substances that may regulate morphogenesis in the embryo.

Angiosperm ovules are bitegmic, unitegmic, or ategmic. The condition of having both outer and inner integuments (bitegmy) is the most prevalent, occurring in 152 dicotyledonous and 52 monocotyledonous families (Bouman 1984). In bitegmic ovules, the outer integument usually affords protection to the ovule, as well as housing the vasculature. The vascularization of the inner integument and nucellus is far less common (Corner 1976). In the outer integument, the vascular pattern can range from a single raphe bundle to an extensive network of reticulating or dichotomizing bundles. The outer integument is often composed of a number of layers of various cell types. The outer layer(s) are generally composed of sclerenchyma cells that afford mechanical protection to the seed. The inner layers are parenchymatous cells of various shapes that usually have numerous air spaces for gaseous exchange. These parenchyma cells may also function in the storage of starch, lipids, and crystals for later utilization by the growing seed.

A unique feature in the ontogeny of the integuments is the differentiation of the endothelial or integumentary tapetal layer. This layer has been observed in 65 dicot and seven monocot families (Bouman 1984). The endothelium differentiates from the innermost layer of the single integument in unitegmic ovules, or from the inner layer of the inner integument in bitegmic ovules. It is generally uniseriate and composed of densely cytoplasmic cells (Kapil and Tiwari 1978). Following degeneration of the nucellus, the endothelium forms the new limiting layer around the embryo sac. The endothelial cells are separated from the embryo sac by a cuticle in Jasione montana (Erdelska 1975), Ornithogalum (Tilton and Lersten 1981), and soybean (present study). The function of this layer is unclear. Because of the cuticle the endothelium has been purported to physically

limit the expansion of the embryo sac. The most important function ascribed to the endothelium is the transfer of nutrients to embryo sac. Engell and Petersen (1977) observed that the character of this layer changes from meristematic to secretory suggesting a metabolically active role in nutrient transport. ATPase activity has been observed at the plasmalemma of endothelial cells of Saintpaulia ionantha (Mogensen 1981), Antirrhinum (He and Yang 1991a), and Helianthus (He and Yang 1991b). These latter authors concluded that the endothelium actively transports nutrients to the embryo sac.

As discussed, recent attention into the development of the endosperm has been generated by the EBN Hypothesis. Plant breeders and geneticists proposed the hypothesis to explain the failure of normal seed set in certain interspecific crosses of a variety of angiosperm species. Generally, the absence of seed formation in some interspecific crosses is not because of an absence of fertilization, but due to endosperm failure and subsequent ovule abortion as found in Trifolium (White and Williams, 1976; Williams and White, 1976). Although, these studies on EBN suggest that the endosperm has an integral role in early embryo development, the nature of endosperm failure and its subsequent role in embryo viability has received little attention in the literature.

The presence of EBN factors in the legume Trifolium (Parrott and Smith, 1986) and preliminary work in Dr. Palmer's lab (USDA-ARS, FCR and ISU Departments of Agronomy, and Zoology and Genetics) suggest that EBN is an agent of early ovule abortion in interspecific crosses of soybean. If EBN is a factor in soybean, we would like to determine the nature of endosperm/embryo interaction which results in ovule abortion in incompatible crosses.

As a preliminary to understanding this interaction, it is first necessary to characterize the development of the embryo, endosperm, and ovule in a normal system and to use this as a model for studying incompatible systems. This study will look at the development of the

soybean embryo, endosperm, and ovular tissues in a normal system as part of a project which addresses the role of these tissues in the development of the embryo.

**PAPER 1: LIGHT AND ELECTRON MICROSCOPY STUDY OF OVULE, ENDOSPERM,
AND EMBRYO DEVELOPMENT IN GLYCINE MAX L. MERR.**

**LIGHT AND ELECTRON MICROSCOPY STUDY OF OVULE, ENDOSPERM, AND
EMBRYO DEVELOPMENT IN GLYCINE MAX L. MERR.**

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ABSTRACT

Various anatomical and ultrastructural aspects of soybean embryo, endosperm, and ovule development are described for the zygote to late heart-shaped embryo stages (0-35 days postfertilization). Nucellar cells subtending the degenerative synergid break down allowing for pollen tube passage to this synergid. In 15 of 17 ovules studied the degenerative synergid occupies a position abfunicular to the zygote. Nucellar cells surrounding the central cell degenerate by wall thinning and accumulation of mucilage exterior to the plasmalemma and in the cytoplasm. The inner integument differentiates into the endothelium and layers of thick-walled cells at the globular embryo stage. The endothelium has a cuticle on its inner surface. Fragmentation of this cuticle parallels the onset of endosperm cellularization. Cellularization of the free-nuclear endosperm is initiated at the globular embryo stage by the formation of anticlinal sheet-like ingrowths of the central cell wall. These walls fuse at their edges to form cylinders. Uninucleate cells are formed within the bases of the cylinders as periclinal walls are laid down. These latter walls are formed in the presence or absence of phragmoplasts. At the onset of cellularization, the endosperm forms a cellular sheath over the apex of the embryo partitioning it from the central cell. The late globular embryo forms a cuticle on its surface but not on the suspensor. The chalazal process remains acellular until the late heart-shaped embryo stage; its cells are suspended in a mucilage due to the loss of the central cell wall in this region. Endosperm degeneration occurs by two different processes: one similar to nucellar degradation; and the other involves the fusion of vesicles to the plasmalemma and the concomitant release of cell wall fibrils. The outer integument differentiates into the palisade epidermis, hypodermis, spongy layers, and a tracheary bar

below the hilum during the heart-shaped embryo stages. The integrated development and function of the above tissues towards embryo and ovule maturation are discussed.

INTRODUCTION

Previous anatomical and ultrastructural studies of the soybean ovule have concentrated particularly on very early stages of development. The events of megasporogenesis and/or megagametogenesis (He et al. 1979; Kennell and Horner 1985; Folsom and Cass 1988, 1989), the prefertilization embryo sac (Folsom and Peterson, 1984; Folsom and Cass, 1990), and various aspects of fertilization in the egg apparatus (Dute et al. 1989) and central cell (Folsom and Cass 1992) have been documented. However, the body of literature on the postfertilization ovule of soybean is fragmented, and only encompasses a short period of development following fertilization. The ultrastructure of the soybean embryo through the proembryo stage was studied by Folsom and Cass (1992) and Dute et al. (1989). Dute and Peterson (1992) characterized the development of the free-nuclear endosperm and the initial stages of its cellularization through the early globular embryo stage. Tilton et al. (1984) and Folsom and Cass (1986) discussed the formation and pattern of embryo sac wall-ingrowths and their role in embryo nutrition.

In these previous studies, little information was presented on the development of the embryo and endosperm beyond the early globular embryo stage. Although some overlap with the latter studies is necessary for descriptive continuity, the intent of this study is to present new information not included in those studies. Our study extends the body of knowledge on the developmental anatomy and ultrastructure of the soybean embryo, endosperm, and ovular tissues from the initiation of the zygote through the late heart-shaped embryo stage (35 days postfertilization). This study is part of a project which addresses the compatibility and interaction of these tissues to the success of the embryo and seed.

MATERIALS AND METHODS

Soybean plants, Glycine max (L.) Merr. cv. Harosoy, were germinated and grown in a glasshouse or in the field. Floral buds or gynoecia, at various stages of development (0–35 days post-fertilization), were placed in 3% glutaraldehyde - 4% paraformaldehyde in sodium cacodylate buffer (0.1M, pH 7.2) at room temperature (22 C, RT). The ovules were dissected in the fixative, placed under vacuum at 15 psi (6.89 kPa) for 1 h, and then placed in fresh fixative at 4 C for 12 h. Fixation was followed by three buffer rinses, postfixation in 1% osmium tetroxide in the same buffer for 4 h at RT, and dehydration in a graded ethanol series. En bloc staining (5% uranyl acetate in 70% ethanol) was done during the dehydration step in some material processed for electron microscopy. Acetone was used as a transitional solvent for infiltration into Spurr's resin (hard recipe). Infiltration in pure resin was accomplished on a rotator over a two-week period (fresh resin daily) before casting.

Light Microscopy (LM)

Resin-embedded material was sectioned at 1 μ m with glass knives on a Reichert Ultracut E microtome and fixed to subbed (poly L-lysine or gelatin) glass slides. A double stain of 0.13% methylene blue - 0.02% azure II in 0.066M NaH₂PO₄ buffer (pH 6.9) followed by 0.2% basic fuchsin in 2.5% ethanol was used for its differential staining properties of thick sections embedded in Spurr's resin. All light microscope observations and photomicrography were made with a Leitz Orthoplan microscope fitted with bright-field, differential interference-contrast, and phase-contrast optics. Images were recorded on Kodak Technical Pan film.

Transmission Electron Microscopy (TEM)

Thin sections (60-90 nm) of resin-embedded material were cut using a Diatome diamond knife and stained with 5% uranyl acetate in 70% ethanol for 1 h, and in aqueous lead citrate for 1 h. Sections were observed and photographed on an Hitachi HU-11C-1 TEM at 75 kV or a Jeol JEM-1200EX-II STEM at 80 kV. Images were recorded on Kodak SO-163 film.

Scanning Electron Microscopy (SEM)

Ovules were fixed and dehydrated as previously described. While in absolute ethanol, ovules were placed in small ethanol-filled cylinders of Parafilm and sealed. The cylinders were immersed in liquid nitrogen for 5 min and the ovules/cylinders fractured on a metal block sitting in a bath of liquid nitrogen. The fractured pieces of ovule were placed in absolute ethanol at 4 C and allowed to come to RT. The ovular material was critical point dried and mounted on brass disks with conducting tape and silver paint. Specimens were coated with gold or gold-palladium in a Polaron Sputter Coating Unit E5100 and observed with a JEOL JSM-35 SEM at 20 kV. Images were recorded on Polaroid 665 film.

RESULTS

Zygote Stage

In soybean (cv. Harosoy), 2-4 ovules are found the length of the upper suture in the horizontally oriented gynoeceium. The ovules are each attached to the gynoecial wall by a short funiculus which has a single collateral vascular strand. This vascular bundle is chalazally positioned within the funiculus, and the phloem is chalazal to the xylem within the strand. The sieve tubes have normal perforation plates and the vessels have spiral wall thickenings. The bundle enters each ovule, via the outer integument, and terminates 3-4 cell layers from the hypostase. Densely cytoplasmic parenchymatous cells (3-4 layers) encircle the vascular bundle. These cells have numerous plasmodesmata and an organellar complement indicative of high metabolic activity: rich in mitochondria, rough endoplasmic reticulum (RER), dictyosomes, and vesicles. The majority of the funicular cells appear less active (highly vacuolate), but store abundant starch in large amyloplasts.

The ovules are bitegmic and campylotropous, the overarching of the outer integument towards the funiculus forms a Y-shaped cleft leading to the micropylar pore. The surface of the outer integument has a cuticle which extends to and lines the micropylar pore. The micropyle is formed only by the outer integument. In medial longitudinal section, the ovule has a differentially thickened outer integument, the adfunicular portion has a greater number of layers than its abfunicular counterpart, 12-16 versus 3-4 cell layers, respectively. In general, the outer integumentary cells are moderately vacuolate and rich in mitochondria, RER, ribosomes and plasmodesmata. Starch-filled amyloplasts, dictyosomes, and vesicles are also common among the outer integumentary cells.

The inner and outer integuments are separated by a cuticle at their chalazal ends. This dense osmiophilic layer appears to be deposited at the interface between both integuments

but extends only a short distance of their combined length. Although the inner and outer integument layers are adnate, no plasmodesmata connect them indicating their ontogenetic separation.

The inner integument is 2-3 layers thick and extends micropylarly from its point of origin along the hypostase to just short of the base of the embryo sac. The cells of the inner integument have numerous organelles, small vacuoles, and plasmodesmata. Amyloplasts are abundant in the micropylar inner integumentary cells. The inner surface of the inner integument is covered with a dense osmiophilic layer, similar to that described between the outer and inner integuments and the surface of the ovule. This cuticularized surface of the inner integument encloses the nucellar tissue laterally, thus only the extreme micropylar and chalazal ends of the nucellus are exposed to the outer integument and hypostase, respectively.

The nucellus is initially composed of densely cytoplasmic isodiametric cells, which are rich in amyloplasts, ribosomes, dictyosomes, and vesicles. These cells also may have transfer cell-like wall ingrowths. Although plasmodesmata are common among nucellar cells, no such connections exist between the nucellus and the integuments or between the nucellus and the embryo sac. As the ovule and embryo sac elongate just prior to and following fertilization, the nucellar cells become elongate and highly vacuolate.

The hypostase is a small group of cells that abuts the chalazal end of the nucellus, and later upon degeneration of the nucellus, borders the chalazal end of the embryo sac. The hypostase cells are cytoplasmically dense and have numerous mitochondria, dictyosomes, amyloplasts, RER, and ribosomes. Individual cells have unevenly thickened walls and the thickenings show greater electron density than adjacent wall material of the same cell.

The embryo sac is conical in medial longitudinal section with the zygote and synergids positioned at its broad micropylar end. In horizontal longitudinal section this sac is hour

glass-shaped. Subtending the base of the embryo sac is a portion of the nucellus consisting of 1-2 layers. A few of the nucellar cells have degraded forming a small gap opposite the degenerate synergid (Fig. 1). This gap exposes the base of this synergid to the outer integuments and micropyle, and thus may be the pathway of pollen tube entrance into the embryo sac. In median longitudinal serial sections of 17 ovules where both the synergids are identifiable, the degenerate synergid is abfunicular and the persistent synergid is adfunicular to the zygote in 15 of them. The pollen tube penetration is associated with the degenerate synergid and its contents are often visible within this synergid (not shown). The pollen tube was not observed exiting the synergid. The degenerate synergid cytoplasm appears as condensed electron-dense material. From the apex of the degenerate synergid is a trail of osmiophilic material which extends to the apex of the zygote and to the primary endosperm nucleus, which is positioned just chalazal to the zygote (Fig. 2). This trail is the same density as that of the degenerate synergid, which suggests that it is the path of the pollen tube nuclei.

The persistent synergid, which is most often adfunicular to the zygote, is cytoplasmically dense, but has a large single chalazal vacuole (Fig. 1). The persistent synergid cytoplasm contains undifferentiated plastids, mitochondria, dictyosomes, RER, and small circular electron dense bodies. The basal filiform apparatus is massive, its digitate walls consisting of material which has a greater electron density than the cell walls of other ovular tissues. The persistent synergid degenerates at the time of the first division of the zygote. Its deterioration is similar to that of its degenerative counterpart; it fills with osmiophilic material, becomes smaller, and then degenerates.

The zygote shows distinct cytoplasmic polarity. Its nucleus and the majority of the cytoplasm are situated at the chalazal end subtended by a large micropylar vacuole (Fig. 2). The cytoplasm contains abundant amyloplasts, mitochondria, ribosomes, polysomes,

dictyosomes, small osmiophilic bodies, and to a lesser extent smooth and rough ER. The zygote initially lacks a cell wall and is bound only by its plasmalemma. The zygote cell wall is laid down shortly after fertilization and is complete prior to the first mitotic division.

Immediately following fertilization, the nucleoplasm of the primary endosperm nucleus is homogeneous and has no inclusions except for two nucleoli of different sizes (Fig. 2). The smaller nucleolus is in a lobe of the primary endosperm nucleus. This lobed portion likely represents the fused sperm nucleus. The fusion is in close proximity to the cytoplasmic trail of the degenerate synergid. In subsequent stages, the primary endosperm nucleus has a single large nucleolus, and is spherical in shape. The first division of the primary endosperm nucleus occurs in the longitudinal plane of the embryo sac. Free-nuclear divisions of the endosperm continue, until there are approximately 16 endosperm nuclei at the time of the first zygotic division.

The wandlabrinthe originates from the micropylar base of the central cell wall prior to fertilization to surround the base of the synergids and zygote. It extends along the central cell wall to approximately one-half the height of the zygote. This digitate mass of wall material consists of regions of varying electron densities (Fig. 3). The more electron translucent parts of the wandlabrinthe are consistent in density to that of the central cell wall while the denser regions are similar to the filiform apparatus. The wandlabrinthe is a persistent feature of the embryo sac, continuing to expand with the enlargement of the ovule through the late heart-shaped embryo stage. The wandlabrinthe becomes homogeneous in density during its subsequent expansion.

The most prevalent feature of the central cell at the zygote stage is the presence of numerous large amyloplasts (Fig. 2). Aggregates of starch completely fill these plastids so that no ultrastructural details of the plastids could be noted except for the outer membrane.

During endosperm development, the starch is rapidly depleted and the amyloplasts are smaller (Fig. 4). These amyloplasts have a translucent stroma, absence of grana and few thylakoids. The central cell, in addition to the amyloplasts, has numerous plastoglobuli, mitochondria, dilated RER and SER, polysomes, ribosomes, dictyosomes, and osmiophilic bodies. The latter structures are usually near the base of the zygote.

Proembryo Stage

At 2-3 days postfertilization, the zygote divides to form the basal and apical cells. Both cells undergo anticlinal followed by periclinal divisions, forming a short torpedo-shaped embryo (Fig. 5). The chalazal-most cells are densely cytoplasmic. The predominant feature of each basal cell is a single large vacuole. The basal-most cells of the proembryo develop transfer cell-like wall ingrowths from their tangential and basal cross walls. As the embryo continues to develop, the wall ingrowths of the basal cells become more extensive. Those walls in contact with the endosperm become particularly elaborate with wall ingrowths.

A large vacuole is formed in the central cell prior to the first division of the zygote and it continues to expand during the enlargement of the embryo sac and ovule. The large central vacuole eventually displaces the endosperm cytoplasm to the periphery of the central cell (Fig. 5). The abundant starch reserves present at the zygote stage are rapidly depleted and are absent from the central cell when the embryo is 16-celled. The thin cytoplasm at the periphery of the central cell also contains the large, free nuclei of the endosperm, mitochondria, dilated RER, ribosomes, and dictyosomes. Cytoplasm associated with the wandlabrinthe also has numerous vesicles and osmiophilic bodies.

The nucellar cells degenerate as the embryo sac expands. The loss of the nucellus does not appear to be solely due to the encroachment of the embryo sac. The nucellar cells demonstrate signs of degradation prior to contact with the embryo sac. The walls become

thinner and an electron dense mucilage-like material accumulates in the wall space that is then secreted into the lumen of the cell by invagination of the plasmalemma. Eventually the organelles become atrophied and there is a general condensation of the cytoplasm. Some of the dense osmiophilic material is noted in the endosperm adjacent to the degrading nucellus, but does not accumulate to any great extent. As the nucellus continues to degenerate, the embryo sac comes in contact with the inner integuments laterally and with the outer integuments at its micropylar base.

The number of cell layers of the outer integument increase as the ovule enlarges, primarily on the adfunicular side. As this side expands, the ovule becomes adnate part of its length with the gynoecium. This adnation continues along the length of the ovule until a distinct hilum is noted at the early heart-shaped embryo stage. Some of the cells of the inner layers of the outer integument form one to several, usually one, Rosanoffian crystals per cell (Fig. 6). The crystals are rectangular in one longitudinal plane and sharply diamond-shaped with two barb-like protrusions radiating from its center in the other longitudinal plane. As each crystal matures, it becomes bounded by a wall-like chamber within the cell. The chamber is usually fused to the inner surface of the cell wall. The outer layer(s) of the inner integument form Rosanoffian crystals similar to those described in the outer integumentary cells. These crystals are found only in the interfacing cell layers of both integuments. No crystal cells are found at the extreme micropylar or chalazal ends of the integuments or in the funiculus. The crystals are readily apparent in these tissues through the globular embryo stage but they are less frequent in subsequent stages. In general, the only visible changes among the integumentary cells from the zygote stage, are a large decrease in the quantity of starch in these cells and an increase in vacuolation.

Globular Embryo Stage

The integuments undergo distinct morphological changes at this stage of development. Two provascular strands form in the adfunicular outer integument. Both strands are connected to the chalazal bundle at a point shortly after it enters the ovule from the funiculus. These strands run the length of the ovule, parallel to each other, and terminate near the micropylar end of the embryo sac.

Opposite the base of the embryo sac and near the terminus of each of these strands are outer integumentary cells that have unusually dense osmiophilic bodies of variable shapes. The origin of these bodies is unclear. Another group of cells with similar dense bodies occurs in the outer integumentary cells just chalazal to and near the terminus of the chalazal vascular strand. The presence of electron-dense material in these regions of the outer integument is persistent through the late-heart shaped embryo stage.

The inner integument differentiates into two distinct tissues (Fig. 7). The outer tissue is composed of 1-7 layers of thick-walled cells each of which has a single large vacuole and a reduced complement of organelles. Although appearing to be metabolically inactive, crystal formation still occurs in these cells. Plasmodesmata are closed off in the cells as their walls thicken. The single inner layer which abuts the embryo sac or nucellus is termed the endothelium or integumentary tapetum. These cells contain many organelles in their dense cytoplasm, but a reduced number of plastids. Plasmodesmata are common between the endothelial cells, but are lacking between this layer and the embryo sac or nucellus. At the inner surface of the endothelium, the cuticle has begun to form thin areas and gaps. The degradation of the cuticle layer occurs at the micropylar end first and proceeds chalazally. The fragmented cuticle disappears during the early and late heart-shaped embryo stages.

At eight days postfertilization, the embryo has differentiated into a globular embryo proper and short suspensor (Fig. 7). The divisions at the apex of the embryo outpace those

of the basal cells giving the embryo its distinctive globular shape. The embryo-proper surface cells divide anticlinally to form a layer of jacket cells. These cells surround an inner core of cells that divide more randomly. The densely cytoplasmic cells of the embryo proper have large nuclei with prominent nucleoli. The cells are also rich in organelles. Late in the globular embryo stage, a thin cuticle forms over the surface of the embryo proper (Fig. 8) but it is absent from the suspensor.

The suspensor cells are larger and more vacuolate than the embryo-proper cells but lack amyloplasts. A distinguishing feature of the suspensor cells is the extensive array of transfer cell-like wall ingrowths on the basal cross and tangential walls of individual cells. These digitate ingrowths are most extensive in those walls in contact with the endosperm. The abundance of these ingrowths per cell decreases acropetally within the suspensor and none are noted in the embryo proper. Vesicles are commonly associated with the wall ingrowths, which suggests that the suspensor cells have a central role in the absorption and transport of materials to and from the embryo. Plasmodesmata are common among the cells of the embryo, but such connections are rare between embryo and endosperm. Only a few plasmodesmata are noted between the basal-most cells of the suspensor and the free-nuclear endosperm.

The endosperm of soybean is of the nuclear-type. It undergoes a number of synchronous free-nuclear divisions prior to cellularization. Cellularization is initiated at the micropylar end of the embryo sac and proceeds chalazally and centripetally (Fig. 7). Also, cellularization of the adfunicular side of the embryo sac precedes that of the abfunicular side. Early in the cellularization of the endosperm a single wall is observed spanning the width of the central cell and it closely covers the apex of the young globular embryo (Fig. 9), thus separating the embryo from the chalazal portion of the embryo sac. The formation of this partitioning wall is unique because further cellularization across the width of the

central cell does not occur until much later. In most cases this ensheathing wall is formed prior to the cuticularization of the embryo proper. Eventually walls will arise from the embryo-ensheathing wall to connect with other endosperm walls (Fig. 7).

Numerous vesicles are observed along the length of the central cell wall, prior to endosperm cellularization. Small amyloplasts, mitochondria each with an electron-dense stroma, RER, ribosomes, normal and highly curved dictyosomes, and small vacuoles are numerous in the endosperm, just prior to cellularization. Peg-like wall ingrowths from the central cell wall extend into its lumen to form anticlinal walls (Fig. 10). These ingrowths often are found opposite the gaps formed in the cuticle of the endothelium. The growth of these walls seems to be a two-part process, the first of which involves the secretion of dense material from the wall itself and, second, the later fusion of dictyosome vesicles. The ends of these growing anticlinal walls form inflated tips (Fig. 11) in which accumulate vesicles filled with material of the same density as the wall (Fig. 10). The vesicles apparently rupture and their contents are released within the inflated tips (Fig. 12). Each inflated tip membrane is an extension of the central cell plasmalemma. The presence of dense material in these bulbous tips suggests that wall precursors are transported through the anticlinal wall and are continuously added to the growing tip. Numerous dictyosomes and their vesicles are present along the lengths of the endosperm anticlinal walls (Fig. 13). The lumens of these vesicles are less dense in comparison to the material found in the inflated tips. Vesicles are observed fused to the plasmalemma near each tip and along the length of each growing wall. These vesicles likely add substrates necessary for completion of cell wall synthesis.

Slender anticlinal walls are observed extending from the existing thicker wall ingrowths of the wandlabrinthe. These walls often are fused to the basal cell walls of the suspensor

(Fig. 14). Only anticlinal walls originating from the wandlabrinthe are observed fused to the suspensor cells.

The surface area of the central cell plasmalemma increases greatly as the anticlinal walls grow into the central cell lumen. The vesicle membranes are incorporated into the plasmalemma as they fuse. Also, multilamellate bodies with stacks of membrane-like material are frequently observed at the junctures of the central cell wall and the newly formed anticlinal walls (Fig. 15). These multilamellate bodies and vesicles may act as sources of membrane material to accommodate the rapid increase in plasmalemma surface area of the central cell.

As the endosperm walls extend into the central cell, they grow in depth, eventually forming sheet-like walls (Fig. 16). The sheets fuse at their ends to form cylinders, which are open centripetally in the central cell lumen (Fig. 17). Once these cylinders of wall material are laid down, completion of individual cells occurs in one of two ways. First the apex of each wall may branch, turn at right angles, or bifurcate to form periclinal walls (Fig. 18). Subsequently, these walls continue to grow and fuse with similar branches growing from the opposing cylinder walls to enclose a single nucleus within a complete cell. This process of wall growth and fusion follows a very regular pattern. At no time are phragmoplasts or microtubules observed during this type of endosperm cellularization. Therefore, it is unclear as to what factors are directing this pattern of cell wall growth and fusion.

In the second type of cellularization, the nucleus within a cylinder undergoes a normal mitotic division with the formation of a phragmoplast (Fig. 19) and subsequent cell wall between daughter nuclei. This newly formed cross-wall fuses with the cylinder wall resulting in the formation of a cell at the base of the cylinder and a free nucleus centripetally at the open end of the cylinder. The combination of both processes of cell

formation creates a single layer of endosperm cells along the periphery of the central cell, first at the micropylar end and continuing chalazally. Once walled in by one of these processes, individual endosperm cells undergo normal mitotic divisions producing daughter cells. All of these various processes, free-nuclear divisions, free-wall growth and branching, cylinder closure after phragmoplast formation, and normal cytokinesis, occur concurrently within the central cell (Fig. 20) until cellularization of the endosperm is complete at the late heart-shaped embryo stage.

An elongate channel is formed at the chalazal end of the embryo sac, which is bound by the nucellus laterally and the hypostase chalazally (Fig. 21). This channel contains large free nuclei embedded in a degenerate cytoplasm lacking in organelles. Dense mucilage-like material is observed throughout the channel and appears to accumulate during the digestion of the surrounding nucellar tissue. The chalazal channel plus the hypostase have been collectively called the endosperm haustorium (Dute and Peterson 1992). This "haustorium" does not actively invade the ovular tissues and, therefore, we prefer to use the term chalazal process. Although not invading the surrounding tissues, cells in contact with the hypostase become necrotic and eventually collapse (Fig. 21).

Early Heart-Shaped Embryo Stage

The cotyledonary primordia are formed 12-15 days postfertilization giving the embryo its distinctive heart shape. The cells of the embryo proper and suspensor are densely cytoplasmic and contain prominent nuclei and small vacuoles. The basal tiers of suspensor cells contain numerous transfer cell-like wall ingrowths as described previously. The cells of the embryo are connected by numerous plasmodesmata. The two young cotyledons lie in the median longitudinal plane of the ovule at this stage of development.

The endosperm is completely cellular except for the chalazal process which remains free-nuclear. The endosperm cells in the central portion of the embryo sac are large, thin-walled and each has a single large vacuole (Fig. 22). These cells possess only a few organelles. Plasmodesmata interconnecting these cells are infrequent. The central endosperm cells rarely divide following cellularization of the endosperm, while the peripheral layers of cells remain meristematic. The divisions of these latter cells keep pace with the expansion of the embryo sac and ovule. Reflective of their mitotic activity, these cells are densely cytoplasmic and rich in organelles. Plasmodesmata are more common between these cells than those more centrally located. The cellular endosperm lacks apparent storage products.

In cross section the outer integument is composed of three regions of tissue. The epidermis of the ovule has differentiated into the palisade layer. The palisade epidermis is two layers thick in the hilar region. The outer layer is contributed by the hilum. These densely cytoplasmic columnar cells have lignified periclinal walls, many times thicker than their anticlinal walls (Fig. 23). Each cell has a large oblong nucleus and is rich in organelles. Numerous layers of highly vacuolate isodiametric cells, below the palisade epidermis, compose the central region of the outer integument. Within these layers differentiates the vasculature of the ovule. The innermost region of the outer integument consists of 6-9 layers of isodiametric cells with numerous intercellular spaces (Fig. 22). These cells are smaller and cytoplasmically denser than the adjacent cells of the outer integument. Dark-staining mucilage-like material fills the intercellular spaces of this region.

The provascular strands described in the globular embryo stage have matured into collateral bundles of xylem and phloem. These bundles are within the central region of the adfunicular outer integument. In the lateral portions of the ovule numerous reticulate

provascular strands differentiate in this central region, as well. These provascular strands are connected to the adfunicular vascular strands at numerous places along their length and extend through the outer integument to the abfunicular side of the ovule. The sieve elements are the first to mature and the xylem elements mature later during the late heart-shaped embryo stage.

The outer layers of the inner integument have differentially changed along the length of the ovule. At the micropylar end, the previously thick-walled cells have stretched and compressed into elongate cells (Fig. 22). Chalazally, these cells retain their earlier morphology but their number of layers has increased. The endothelium remains unchanged.

Late Heart-Shaped Embryo Stage

The embryo forms a distinct dome-shaped shoot apical meristem (Fig. 24) approximately 20 days postfertilization and the radicle meristem is formed shortly after. The embryo fills the micropylar half of the embryo sac with its large cotyledons. The hypocotyl is well developed and consists of the protoderm, cortical ground meristem, and the procambium. The cotyledons expand laterally to form their ab- and adaxial surfaces. The plane of these surfaces is at right angles to the sides of the ovule. As the embryo grows it gradually rotates (Fig. 25) 90 degrees so that the surfaces of the cotyledons are now parallel to the sides of the ovule. This is the position that the cotyledons will have in the mature seed. Subsequent to the formation of a procambium in the hypocotyl, procambial tissue is formed in the cotyledons. As the cotyledons continue to develop numerous anastomosing provascular strands are formed throughout their ground tissues. Upon differentiation of these vascular strands, the cortical cells begin to fill with starch and large spherical protein bodies. During subsequent stages of development small dense bodies, likely lipid, are observed in these cells as well.

The suspensor is longer than in previous stages, due to the elongation of its cells (Fig. 24). Only the basal tier of suspensor cells have transfer cell-like wall ingrowths and the latter are especially prominent at the tangential walls interfacing the endosperm and on the basal cross walls. The late heart-shaped embryo has a thin cuticle on its surface, but unlike earlier stages it extends around the suspensor. Only the basal tier of cells lacks a cuticle.

The integuments begin to take on the characteristic morphology of the mature testa. Centripetally, the testa is composed of five layers beginning with the palisade epidermis (Fig. 26). The cells of the palisade layer fill with electron dense spherical bodies as they mature. These bodies are similar to those formed in the outer integumentary cells opposite the micropylar end of the embryo sac. The hypodermis is a single layer of osteosclereids or "hour-glass" cells with lignified anticlinal walls. Next is a spongy tissue composed of several layers of lacunate parenchyma with large tangentially elongate lobes. The vasculature of the ovule and the tracheary bar (Fig. 27) are within this layer of tissue. The latter runs from near the micropyle to midway up the length of the ovule below the hilar groove. The tracheary bar is tear drop-shaped in cross section with its narrow apex exposed to the surface of the hilum. This structure is composed of numerous swollen xylem elements (tracheoids) with reticulate wall thickenings (Fig. 27). The outer layers of the inner integument are crushed during the expansion of the ovule and only a remnant of these thick-walled cells remain (Fig. 25). The endothelium remains unchanged, but it too is eventually crushed during ovular growth.

The endosperm degenerates as the embryo expands. No physically ruptured endosperm cells were observed near the embryo. The endosperm degrades by two morphologically distinct processes which cause the progressive thinning of the endosperm wall. One process occurs in those cells near the base of the suspensor and is initiated by the fusion of dictyosome vesicles to the plasmalemma at the inner surface of the endosperm cell wall

(Chamberlin et al. 1973b) These cells also have numerous mitochondria, ribosomes, and RER in close proximity to this region of the plasmalemma. Long slender fibrils slough off from the exterior of the cell wall opposite the regions of plasmalemma with fused vesicles. This fibrillar material is of the same electron density as that of the cell wall itself and, therefore, may be cellulosic fibrils. Subsequently, these free fibrils aggregate intercellularly to form a mucilage-like material of greater electron density than that of the walls.

The second process of endosperm degeneration occurs predominantly in endosperm cells of the chalazal half of the embryo sac. This process is similar to that observed in the nucellar cells and involves the formation of electron dense mucilage-like material in the space between the wall and plasmalemma of the cell (Fig. 28). This material appears to be deposited by dilated RER fused to the plasmalemma. Coincident with mucilage accumulation in the wall space, the cell wall deteriorates. Mucilage-filled vesicles are formed by invaginations of the plasmalemma and they accumulate in the cytoplasm. These vesicles eventually rupture and the mucilage diffuses into the cytoplasm (Fig. 28). This process continues until the cell wall is completely degraded and the cytoplasm becomes condensed.

The chalazal process elongates with the expansion of the ovule. In light micrographs, the now cellular endosperm of the process appears to have thick walls delineating them from the more micropylar thin-walled endosperm cells. The former cells are thinned-walled, but they stain strongly due to the presence of electron-dense material between the cell wall and plasmalemma. This dense material in the cell wall space is similar to that described for degenerating endosperm cells. These cells are atrophied and have few organelles. The extreme chalazal end of this process has a sparse number of endosperm cells embedded in a dense granular matrix (Fig. 29). The endosperm cells are unattached and suspended in this matrix due to the absence of the central cell wall (Fig. 30). The loss

of this wall is likely due to its rupturing during the rapid elongation of the ovule. In the absence of a defining wall, the chalazal-most end of the process is bound laterally by only the endothelial cells.

DISCUSSION

Because the ovule of soybean is crassinucellate, the pollen tube must penetrate intervening layers of nucellus between the micropyle and embryo sac. Nucellar cells degenerate micropylar to the abfunicular synergid providing a path for the passage of the pollen tube into the embryo sac. Prior to fertilization in cotton, a similar group of nucellar cells degenerate to accommodate the passage of the pollen tube (Jensen 1969). The dissolution of these cells may be promoted by secretions of the soybean degenerate synergid that breaks down just prior to fertilization (Tilton 1981; Dute et al. 1989). In addition to a pathway for pollen tube entrance, the degradation products of these nucellar cells may have a chemotropic effect in pollen tube attraction. In Paspalum, Choa (1977, 1979) observed a PAS-positive substance in the micropyle involved in the guidance of the pollen tube. He concluded that enzymes secreted by the synergids were involved in the dissolution of integumentary cells and their conversion into a mucopolysaccharide attractant. The synergids as secretory cells involved in pollen tube attraction have been suggested in a number of diverse taxa (Tilton 1981).

In 15 of 17 ovules observed in this study, the abfunicular synergid degenerated first. The first degenerating synergid consistently contained the pollen tube and its contents as noted in numerous taxa (Mogensen 1984) and in this study. Jensen (1974) suggested that this degenerate state is a requisite condition for pollen tube penetration. This preference for degeneration of one synergid over the other has not been shown in other soybean genotypes (Dute et al. 1989; Folsom and Cass 1992), but has been observed in barley (Mogensen 1984), Linum usitatissimum (Russell and Mao 1990), and Helianthus (Yan et al. 1991).

Why one synergid generally degrades before the other is unclear. Morphological data on the two synergids does not give a clue. The synergids of soybean (Folsom and Cass 1990), Solanum (Briggs 1992), Brassica (Sumner and Van Caeseele 1989) and other taxa (Tilton 1981) appear to be cytologically equal. Since this is integral to understanding fertilization in angiosperms, physiological and histochemical studies along with quantitative studies, are warranted to understand the nature of this selective degeneration.

Electron-dense material extruded from the chalazal end of the degenerative synergid partially covers the apex of the zygote. This extrusion is optimally situated distal to the egg and next to the secondary endosperm nucleus for facilitating double fertilization and marks the pathway of the sperm nuclei.

The zygote has the same polarity and similar organellar content as that seen in other soybean genotypes (Dute et al. 1989). The primary endosperm nucleus has a large and small nucleolus embedded within an amorphous nucleoplasm. The smaller nucleolus likely originated from the sperm nucleus because it is within a lobe nearest to the path of the extruded material of the synergid.

The wandlabrinthe is a conspicuous outgrowth of the micropylar central cell wall, surrounding the base of the zygote and synergids. This labyrinth of wall ingrowths is a common feature in a number of diverse taxa such as Helianthus (Newcomb and Steeves 1971), Eschscholzia (Negi 1972), Stellaria (Newcomb and Fowke 1973), Euphorbia (Gori 1977), Proboscidea (Mogensen 1978), and Capsella (Schulz and Jensen 1973). It is common in the legumes Pisum (Marinos 1970), Vicia, Phaseolus, Spartium, and Lathyrus (Gunning and Pate 1974), as well. The wandlabrinthe is a mosaic of wall material of different electron-densities as observed in the present study. We believe that the different densities are due to the deposition of very dense wall material from the filiform apparatus as the synergids degenerate and this is deposited upon the wandlabrinthe as it is forming.

In soybean, the wandlabrinthe may facilitate the transport of solutes into the embryo sac from the integuments near the micropyle (Tilton et al. 1984). The wandlabrinthe is analogous to the wall ingrowths of transfer cells. These latter cells are shown to have elevated levels of ATPase activity suggesting an active transport mechanism (Brentwood and Cronshaw 1978). In an autoradiographic study utilizing labeled $^{14}\text{CO}_2$, the wandlabrinthe appears to concentrate label from the micropylar outer integuments which is then transported to the tissues at the base of the embryo sac (Chamberlin et al., accepted). In the present study, we observed transfer cell-like wall ingrowths in the nucellus, in the lateral wall of the central cell, base of the proembryo, suspensor of the globular embryo, and basal suspensor cells of the heart-shaped embryo. It seems likely that these ingrowths function similar to the wandlabrinthe in transport of solutes by increasing the surface area of the cell. During cellularization of the endosperm, we noted anticlinal walls from the wandlabrinthe fused to the base of the suspensor. These may act as direct apoplastic channels of nutrient transport. It also has been suggested that the filiform apparatus may absorb and transport metabolites to and from synergids (Pritchard 1964; Jensen 1965; Schulz and Jensen 1968; Cass and Karas 1974; Philipson 1977).

More than simply housing the zygote, the central cell is dynamic in its postfertilization development. This is suggested by its organellar complement, transfer cell-like wall ingrowths, rapid divisions of the free-nuclear endosperm, and breakdown of its starch reserves. According to Folsom and Cass (1992), vacuoles fuse with the multigrain amyloplasts and aid in their breakdown. This pattern has been noted in the endosperm of a number of taxa. Subsequently, these vacuoles coalesce into the large central vacuole which occupies the space left by the amyloplasts (Folsom and Cass 1992). The hydrolysis of the starch reserves likely forms a pool of water-soluble carbohydrates in the central cell that could be readily absorbed by the free-nuclear endosperm. This rich carbon source also is

accessible to the young embryo as indicated by plasmodesmatal connections between the central cell and zygote, proembryo (Dute et al. 1989), and the early globular embryo (present study).

Some interpretation must be given to the size of the central cell. Cells of this size are virtually unprecedented in land plants. The thin central cell wall does not likely afford much structural integrity against the physical pressures exerted by the expanding ovular tissues. To keep the cell from collapsing an opposing pressure must build up internally which would provide rigidity to the central cell wall. As the starch reserves break down in the central cell, it is presumed that its solute concentration and osmotic pressure increases relative to the external tissues. Water likely diffuses into the central cell from the integuments to equilibrate this difference in osmotic pressure and, thus, give the central cell sufficient turgor to maintain its large size. The regulator of osmotic pressure in the central cell is likely the large central vacuole. In Haemanthus, as the osmotic value of the central vacuolar sap increases there is a concomitant increase in the size of this vacuole (Ryczkowski 1960).

The central cell starch aggregates are completely degraded during the late proembryo stage and, therefore, it may be assumed that the solute concentration is depleted rapidly. Through the early globular embryo stage, the central cell continues to expand until it is approximately 6000-fold larger in volume than single cells of the adjacent endothelial layer. It is now unlikely that nutrient flux into the central cell is sufficient enough to maintain the solute concentration and osmotic pressure necessary to sustain turgor pressure within the central cell. Why then does the central cell not collapse? The answer may lie in the cellularization of the endosperm which occurs during the early globular embryo stage. The initial wall ingrowths may add structural integrity to the central cell wall. As cellularization continues, the coenocytic central cell is partitioned gradually into smaller

endosperm cells which would relieve the necessity for high turgor pressure in the central cell. It is unclear if the soybean central cell would collapse in the absence of turgor pressure created by a high solute concentration within it. Indeed, we can only presume that a high solute concentration exists in the central cell of soybean as it does in the Phaseolus vulgaris central cell (Smith 1973). It also has been suggested that a high osmotic environment in the central cell may stimulate nutrient uptake by the embryo (Folsom and Cass 1992). Norstog and Klein (1972) believe that high osmotic pressure in the central cell may be necessary to prevent precocious germination of the embryo.

Divisions of the free-nuclear endosperm precede those of the zygote. These divisions are rapid and synchronous as indicated by the number of nuclei in the same phase of karyokinesis in any one section. This synchronism of free-nuclear endosperm divisions has been observed in wheat as well (Bennett et al. 1973). Gunning and Steer (1975) noted that nuclei sharing a common mass of cytoplasm commonly have synchronous divisions. Following a period of rapid divisions, the free-nuclei of the endosperm line the periphery of the soybean central cell. Upon enzymatic digestion and removal of integuments to expose the embryo sacs, Chamberlin et al. (1993) observed that the endosperm nuclei were evenly spaced throughout the central cell, except for their sparse nature in the chalazal process. The rapid synchronous divisions and the nonrandom position of the nuclei implies that there is a regulatory mechanism involved in the divisional process and positioning of the endosperm nuclei. An immunocytochemical study in wheat has shown that the endosperm free-nuclei are interconnected by a cytoskeleton of tubulin (Van Lammeren 1988). Microtubules were shown to radiate from the nuclei that anchor the nuclei in stage- and locus-specific positions in the central cell. The interconnection of the endosperm nuclei suggests that there is a communication between these nuclei that is responsible for their synchronous divisions and short-distance movement.

Cellularization of the soybean endosperm is initiated by the formation of anticlinal wall-ingrowths from the central cell wall. The tip of each of these walls grows in the absence of microtubules, similar to that seen in Stellaria (Newcomb and Fowke 1973) and Haemanthus (Newcomb 1978). But this is in contrast with reports of apical wall growth in the presence of microtubules in other soybean genotypes (Dute and Peterson 1992), wheat (Mares et al. 1977), cotton (Jensen et al. 1977), and Arabidopsis (Mansfield and Briarty 1990). We do not believe that our different results, when compared to other soybean genotypes, are a genotype-specific trait or that they were due to poor tissue preparation in that microtubules were noted in other stages of endosperm development and in other tissues. A possible explanation could be that apical growth of these walls is not a continuous process and that during periods of growth cessation the microtubules are absent.

After a period of growth, the anticlinal walls grow laterally to form sheet-like walls that then fuse at their edges to form cylinders open to the central cell distally. These walls branch periclinally then fuse to enclose a single nucleus within each now complete cell. This pattern of cross-wall formation by branching and fusion is observed in Stellaria (Newcomb and Fowke 1973) and in the first four peripheral layers of endosperm in Haemanthus (Newcomb 1978). In the present study, the formation of the peripheral layer of endosperm utilizes a second and less frequent type of periclinal wall formation within the cylinders. This involves the formation of a phragmoplast between sister nuclei after karyokinesis. This pattern is the sole method by which these cross-walls are formed as reported by Dute and Peterson (1992) for soybean and in Helianthus (Newcomb 1973), and wheat (Mares et al. 1977; Fineran et al. 1982; Van Lammeren 1988). In soybean, once a nucleus has been compartmentalized by one of the two latter processes, it now undergoes normal mitotic divisions with the formation of a phragmoplast. Although, various patterns of wall formation exist among different taxa, and even within a single embryo sac (present

study; Newcomb 1978), normal mitotic divisions following compartmentalization seem to be the only consistent pattern among the previously mentioned studies.

Three distinct patterns of endosperm wall formation were noted in the present study: 1) wall ingrowths of the central cell wall that grow and branch in the absence of microtubules, 2) phragmoplast-aided formation of cross-walls between sister nuclei within prior formed cylinders, and 3) normal mitosis within already compartmentalized cells. It is curious that all three of these types of wall formation can occur at the same time within a given central cell. This would suggest a highly coordinated regulatory system in a seemingly chaotic coenocytic central cell. In wheat, the free-nuclei of the endosperm are interconnected by cytoskeletal elements (Van Lammeren 1988). A similar association of elements may exist in the endosperm of soybean. These connecting elements may aid in coordinating the biosynthetic machinery of the numerous nuclei and, therefore, orchestrate the cellularization of the endosperm.

In the present study, anticlinal wall-ingrowths of the central cell wall lack a phragmoplast-like mechanism at their growing tips similar to that seen in wheat (Mares et al. 1977). It is therefore, unclear how these walls are synthesized. Immunocytochemical studies of plant cell wall biosynthesis have shown that wall matrix polysaccharides, such as pectins and hemicelluloses, are formed at the dictyosomes, but priming reactions probably occur at the ER (Brett and Waldron 1990). In the soybean endosperm, these organelles are in close association with the growing tips of the wall. It is likely that dictyosome vesicles transport these matrix polysaccharides to the wall where they fuse with the plasmalemma and are integrated with cellulose in the wall. Available evidence suggests that cellulose is formed at or just outside the plasmalemma (Brett and Waldron 1990). In the present study, gaps in the endothelial cuticle frequently are observed opposite the base of these growing walls. These interruptions in the cuticle allow the direct absorption of nutrients from the

integumentary tissues via these wall ingrowths in Capsella (Schulz and Jensen 1974). It is possible that these nutrients travel apoplastically within the soybean anticlinal wall and are deposited at its plasmalemma. It may be speculated that the carbohydrate fraction of these nutrients is converted into cellulose which is extruded into the plasmamembrane swelling at the tip of the growing wall. The unused nutrients are likely packaged into vesicles and released into the cytoplasm as a nutrient source for the central cell.

Cellularization of the endosperm proceeds rapidly in the micropylar three-quarters of the embryo sac, but the chalazal channel remains acellular until the late heart-shaped embryo stage. The hypertrophied nuclei and the lack of organelles suggests that this channel is physiologically distinct. Autoradiographic evidence indicates that labeled assimilates from the chalazal vascular strand enter the channel via the hypostase and move unimpeded through the channel's common cytoplasm to the embryo sac (Chamberlin et al. accepted). Indeed numerous studies have suggested that the free-nuclear condition of the chalazal process is essential to its function in the translocation of nutrients to the embryo sac as cited in reviews by Maheshwari (1950), Chopra and Sachar (1963), Bhatnagar and Sawhney (1981), and Vijayaraghavan and Prabhakar (1984). In interspecific crosses of Trifolium, there seems to be a correlation between the absence or abnormal development of the chalazal process and ovule abortion (Williams 1987). In the soybean hypostase the cells appear to be cytoplasmically active and have numerous plasmodesmata suggesting their role in the active transport of nutrients. Similar conclusions can be drawn about the hypostase cells in Solanum (Briggs 1992) with the presence of transfer cell-like wall ingrowths and in Antirrhinum with high ATPase activity (He and Yang 1991). Endosperm cellularization in the chalazal channel of soybean likely precludes its function in nutrient transport as evidenced by the nutritionally deprived necrotic condition of these cells.

The cellular endosperm of soybean has no conspicuous forms of storage products and seems to have no direct role in nutrifying the embryo. But the endosperm does appear to translocate nutrients into the embryo sac via the common apoplast of its cell walls as suggested by autoradiographic evidence (Chamberlin et al. accepted). Nutrients from the integuments are likely channeled through the transfer cell-like wall ingrowths of the lateral central cell wall and the wandlabrinthe. The endosperm as a mechanism of nutrient transport to the embryo sac has been suggested in Pisum (Marinos 1970), Quercus gambelii (Singh and Mogensen 1976), and Zea mays (Schel et al. 1984).

As the endosperm degenerates, it leaves a void outlining the apex of the embryo proper. This suggests that the embryo secretes enzymes responsible for the endosperm digestion. The presence of a cuticle at the surface of the embryo proper and the lack of secretory activity (high vesicle content) within its cells indicates that this is not the case. Indeed, the endosperm cells near the apex of the embryo appear to be autolytic and digest from the inside out. A similar autolysis was noted in lettuce endosperm (Jones 1974). The presence of dense mucilage-like material in the wall space along the plasmalemma and the coincident thinning of the endosperm walls seems to justify this conclusion. The coincident accumulation of osmiophilic material in the wall space and the degeneration of the endosperm also has been observed in Zea (Schel et al. 1984) and Euphorbia (Gori 1987). The dense material accumulates in the lumen of the soybean endosperm cell with the eventual rupturing of the cell. This material once released does not accumulate to any great extent in the embryo sac. It is presumed to be a form of soluble carbohydrate that is absorbed by the embryo. Weakening of endosperm by enzymatic degradation of selected wall constituents (mannans) has been observed in tomato (Groot et al. 1988) and Datura (Sanchez et al. 1990).

During the free-nuclear endosperm stage, the nucellar cells show the same pattern of degeneration as that of the endosperm cells, but without physical breakage due to the encroachment of the central cell. In barley, it is suggested that the nucellus degenerates in a autolytic manner as well (Norstog 1974). The electron-dense mucilage material does accumulate along the chalazal end of the soybean central cell wall. Since no significant accumulation of this material is observed during cellularization of the endosperm it is assumed to have been absorbed by the endosperm. The similar autolytic manner in which the endosperm and the nucellar cells degenerate with the formation of electron-dense material in the wall space suggests a similar catabolic pathway possibly regulated by the central cell.

The endosperm cells near the suspensor degenerate in a different manner than those cells more chalazal in the embryo sac. The embryonic cuticle is absent from the basal cells of the suspensor suggesting that these could have a more intimate role in endosperm digestion. But as in those cells of the embryo proper, the suspensor cells lack the cytology indicative of a secretory function. In the endosperm cells, the presence of numerous dictyosome vesicles fused to the plasmalemma and the concomitant release of fibrillar material from the exterior of the wall indicate an autolytic process. We believe these are cell wall polysaccharides released by hydrolytic enzymes synthesized in the endosperm cells. Outside the cell, the fibrils appear to condense and are transformed into a mucilage-like material. Because there is no appreciable accumulation of this material in the embryo sac, it is likely absorbed by the suspensor cells. This material is similar to galactomannans, which are mucilaginous storage polysaccharides, common in the endosperm cell walls of some legumes (Reid 1985). Therefore, although the soybean endosperm cells have no storage products in their lumens, the embryo seems to receive some nutritional benefit from

the cellular endosperm by absorbing the mucilage-like material formed during the degeneration of the endosperm walls.

The early ontogeny of the soybean embryo is likely dictated by the cytoplasmic polarity of the zygote. The distribution of the nucleus and the majority of the cytoplasm in the chalazal end and vacuolate nature of the micropylar end of the zygote foreshadows the course of embryogenesis. The first division (transverse) of the zygote forms the densely cytoplasmic chalazal cell which gives rise to the embryo proper and hypophysis, while the vacuolate micropylar cell forms the suspensor. This polarity is reflected in the later stages of embryo development as well. The embryo proper is composed of densely cytoplasmic cells, while the suspensor cells are more vacuolate. The cytoplasmic polarity of the zygote seems to be a consistent feature in other angiosperm taxa (Natesh and Rau 1984).

According to Yeung and Clutter (1978) the cytology of the basal cells of the Phaseolus proembryo and the suspensor of later stages suggests an active absorptive function. These authors indicate that the pattern of numerous small vacuoles in these cells parallel that of premature nectary cells, and that both cell types are actively sequestering solutes from surrounding tissues. The pattern of vacuolation in the proembryo basal and suspensor cells suggests a similar function in soybean. These nutrients are likely supplied via the wandlabrinthe which encircles the base of the embryo or from the central cell which has numerous starch-filled plastids. Other features that indicate that these cells are involved in nutrient uptake are: 1) the presence of plasmodesmatal connections between the central cell and proembryo (Dute et al. 1989; Folsom and Cass 1992), and suspensor of the globular embryo (present study); 2) the proliferation of transfer cell-like wall ingrowths in the basal cells of the proembryo and suspensor; and 3) the accumulation of labeled photoassimilates in the proembryo basal cells and later, the embryo suspensor cells (Chamberlin et al. accepted).

Solutes also may diffuse into the embryo from the amyloplast-rich central cell. The zygote and early embryo are immersed within liquid endosperm indicating that the nutrients of the central cell are readily accessible to the zygote and embryo over their entire surface. During cellularization of the endosperm, the early globular embryo is partitioned from the rest of the central cell by the formation of an ensheathing endosperm wall and eventual cellular endosperm. Similar endospermic sheaths surrounding the embryo have been observed in Phaseolus coccineus (Yeung and Clutter 1978) and Pisum sativum (Marinos 1970). These authors suggested that the sheath regulates the flow of nutrients from the liquid endosperm to the embryo. The latter author suggests that the sheath serves to maintain the orientation of the embryo. The formation of the ensheathing endosperm likely precludes the access of these nutrients to the embryo and mandates that the embryo receive its nutrients from the micropylar end of the embryo sac.

The globular embryo forms a cuticle over its surface generally after the formation of the ensheathing cellular endosperm. But the cuticle is absent from the suspensor. The absence of a cuticle and the pattern of wall ingrowths in the suspensor indicate that the suspensor absorbs nutrients from the micropylar base of the embryo sac and these are channeled acropetally to the embryo proper. This is supported by autoradiographic evidence (Chamberlin et al. accepted). The cellular endosperm surrounding the embryo proper lacks apparent nutrients. Therefore, cuticularization of the embryo proper may be a consequence of the partitioning of the embryo from the central cell, thus limiting the outflow of nutrients from the embryo proper to the nutrient poor cellular endosperm. At the late heart-shaped embryo stage the cuticle covers the surface of the embryo except for the basal-most tier of suspensor cells. The suspensor remains as the primary route of nutrient flux to the embryo (Chamberlin et al. accepted). The presence of an embryonic cuticle suggests that the embryo is becoming more autonomous in its development toward

maturation. The division of labor in the embryo, exemplified by the sequestration of nutrients via the suspensor, is evidence of this.

In horizontal longitudinal sections the embryo sac is constricted midway up its length giving it an hour glass-shape. While in median longitudinal section the embryo sac is cone-shaped with the chalazal end attenuated. At the early heart-shaped embryo stage the cotyledons are initiated in the median longitudinal plane. Due to the subsequent lateral expansion of the cotyledons, the embryo gradually rotates 90 degrees to accommodate the passage of the cotyledons through the constriction in the embryo sac. This rotation also allows the cotyledons to better fit the broader chalazal end of the embryo sac in horizontal longitudinal section. The shape of the embryo sac likely dictates the rotation of the embryo and the final position of the cotyledons. But in the present study we were unable to determine why such a shape has evolved in light of the necessity of the growing embryo to rotate.

At 20 days postfertilization most of the tissue systems of the hypocotyl are laid down and consist of the cortical ground meristem, protoderm and the procambium. The root initials are formed just above the attachment of the suspensor and a broad apical dome is conspicuous between the cotyledons. The procambium of the cotyledons forms subsequent to and becomes continuous with the procambium in the hypocotyl. At 30 days postfertilization the reticulate vasculature of the cotyledons differentiates and their cells begin to fill with storage reserves soon after.

Differentiation of the integuments first occurs in the inner integument with the formation of the endodermis and layers of thick-walled cells at the early globular embryo stage. The formation of the endodermal layer at an early stage of ovular development, and its persistence through the late heart-shaped embryo stage suggests that this layer has a role in the development of the adjacent embryo sac. The abundance of

mitochondria, dictyosomes, and rough endoplasmic reticulum in the dense cytoplasm of the endothelium suggests a secretory tissue, probably involved in the transport of nutrients to the embryo sac. A similar function has been attributed to this layer in Bellis perennis (Engell and Petersen 1977), and Saintpaulia ionantha (Mogensen 1981). The latter study localized ATPase activity at the plasmalemma of the endothelial cells which suggests a metabolically active transport process. The thick-walled cells of the inner integument likely play a supportive role in embryo sac nutrition by protecting the endothelium from being crushed by the differentially expanding outer integuments. Indeed these thick-walled cells are crushed well before that of the endothelium.

The endothelial cuticle is likely a barrier to solute movement into the embryo sac at the early stages of ovule development. The degeneration of this cuticle occurs at a time when the embryo sac is a significant nutrient sink during the cellularization of the endosperm and rapid growth of the embryo. Autoradiographic evidence indicates that nutrients pass into the embryo sac at the onset of degeneration of this barrier (Chamberlin et al. accepted).

It is curious to note that the outer surface of the outer integument, including the micropyle, has a cuticle but only a remnant of a cuticle exists between the outer and inner integuments. During the evolution of the soybean ovule this latter cuticle was likely reduced to allow the passage of solutes from the vasculature of the outer integument to the inner integuments and embryo sac. The persistence of the endothelial cuticle during the evolution of the soybean ovule suggests the importance of this trait to successful maturation of the soybean seed.

The initial vascularization of the outer integument coincides with the breakdown of the endothelial cuticle. Since there are no vascular connections with the inner integuments,

nutrients from the vasculature likely diffuse down a concentration gradient towards the endothelium before entering the lateral regions of the embryo sac (Thorne 1981).

The major tissues of the testa, which is composed of outer integument only, are discernible at the late heart-shaped embryo stage. The palisade epidermis functions in mechanical protection of the seed as suggested by its thick lignified periclinal walls. The tightly packed columnar cells and the presence of a cuticle at its surface indicates that it also is impermeable to water. The high degree of water impermeability found in seed coats seems to be related to the deposition of phenolics in their cells (Marbach and Mayer 1974). In the present study, dense osmiophilic material accumulated within the palisade cells at the late heart-shaped embryo stage. Although not identified chemically, this material could be phenolic compounds and function similarly in the impermeability of the soybean seed coat.

The adfunicular region of the soybean ovule is much thicker than its abfunicular counterpart, due to the presence of the hilum, two main veins, and tracheary bar. In legume taxa it is reported that the palisade layer of the seed is impermeable to gaseous exchange and that gases enter the seed through the valvular action of the hilum (Hyde 1954). Because the tracheary bar is exposed to the hilar surface it is likely involved in gaseous exchange once the seed has abscised from the funiculus. Its large surface area in contact with the inner spongy parenchyma layers of the outer integument suggests this, as well. The cells of the hypodermal layer of the ovule seem to be optimally constructed for support and gaseous exchange. The lignified anticlinal walls are highly constricted forming an expansive void for the tangential movement of gases.

In conclusion, the growth and differentiation of the soybean ovule, embryo sac, and embryo occur in a series of interdependent stages and follow a characteristic pattern. In light of the fact that the embryo, endosperm, and ovule are three genetically distinct sets of tissue(s), their development and interaction must be highly orchestrated. This is

exemplified in the soybean ovule, where numerous tissues are successfully integrated in their actions to support the development of the embryo and bring about the formation of a viable seed.

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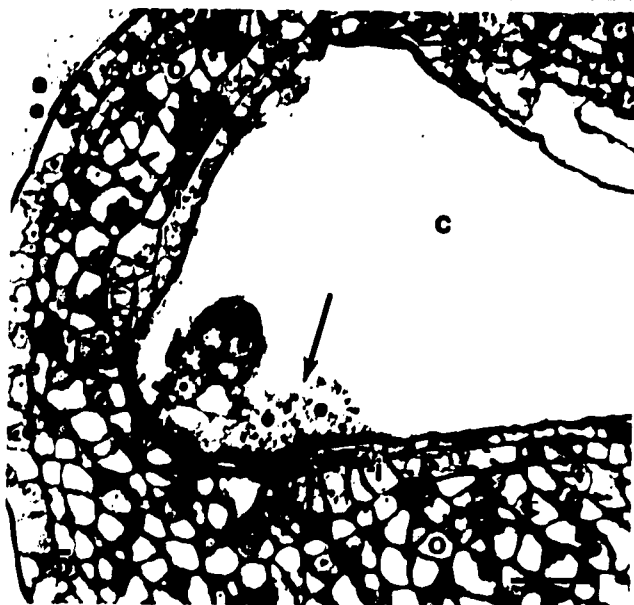
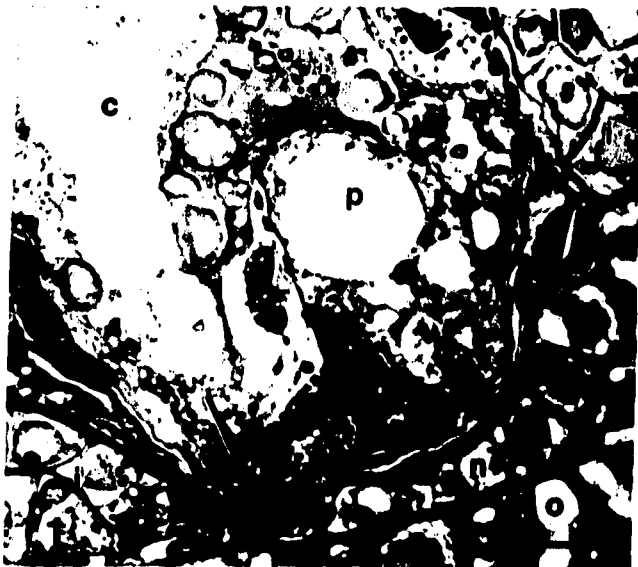
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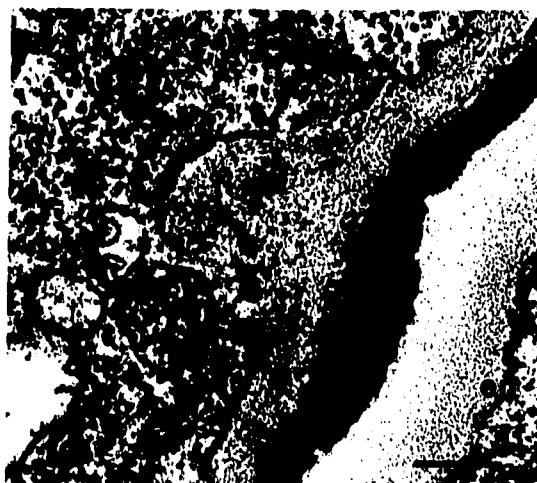
We thank Bruce Wagner for his assistance in the use of the electron microscopes. A special thanks to Dr. Nels Lersten for his wealth of knowledge and feedback in preparing this manuscript.

APPENDIX

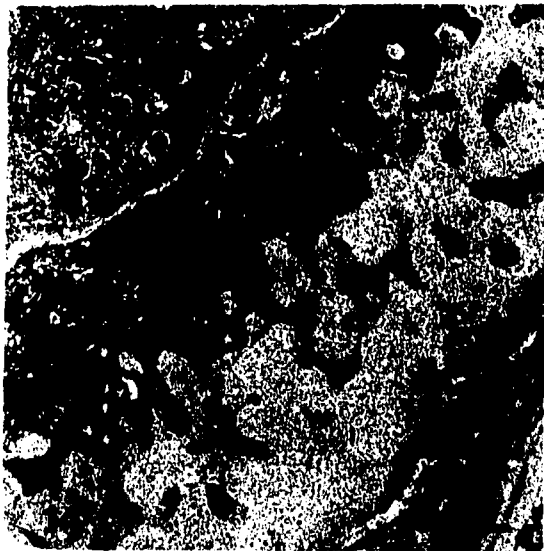
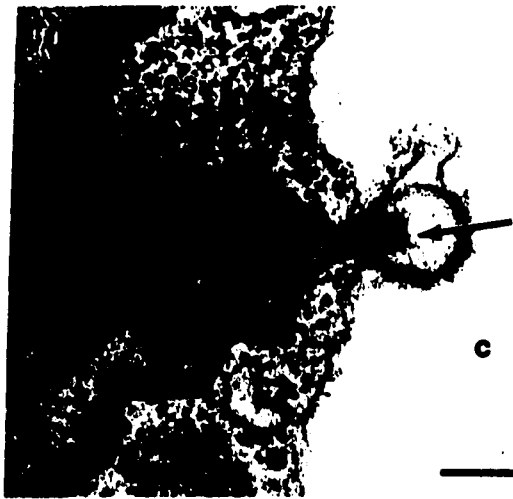
Figs. 1-6 Soybean zygote (figs. 1-4) and proembryo (figs. 5, 6) stages. Fig. 1, micropylar end of embryo sac with persistent (p) and degenerate (d) synergids. Note breakdown of nucellar cells (n) at arrow micropylar to degenerate synergid. c = central cell vacuole; i = inner integument; o = outer integument, bar = 10 μ m. Fig. 2, persistent (p) and degenerate (d) synergids subtending zygote (z). Note trail of dense material (arrows) leading from the degenerate synergid to apex of zygote and primary endosperm nucleus (es). Asterisks (*) denote aggregates of starch in central cell, bar = 10 μ m. Fig. 3, wandlabrinthe (w) displays different wall densities. c = central cell, bar = 1 μ m. Fig. 4, following fertilization amyloplasts (*) lose their starch, become smaller and have no grana, bar = 1 μ m. Fig. 5, micropylar end of central cell (c) showing proembryo and two adjacent free endosperm nuclei (arrow). Central cell vacuole is enlarged. i = inner integument; o = outer integument, bar = 20 μ m. Fig. 6, Rosanoffian crystals and crystal cells in outer integumentary cells. Note crystal chambers are fused to cell walls, bar = 10 μ m.



Figs. 7-11 Globular embryo stage. Fig. 7, embryo with young suspensor (s) surrounded by developing cellular endosperm (es). c = central cell vacuole; i = inner integument; o = outer integument, bar = 20 μ m. Fig. 8, cuticle (arrow) on surface of embryo proper (e). es = endosperm, bar = 1 μ m. Fig. 9, ensheathing endosperm (es) wall (arrow) covers apex of embryo (e), bar = 1 μ m. Fig. 10, peg-like wall ingrowth of central cell wall with inflated tip during the initial stages of endosperm (es) cellularization. Note vesicles within inflated tip. en = endothelium, bar = 0.2 μ m. Fig. 11, inflated apex of growing endosperm (es) wall. Bulbous growth tip surrounded by central cell plasmalemma (arrow) and central cell vacuole (c) tonoplast (arrowhead), bar = 0.2 μ m.



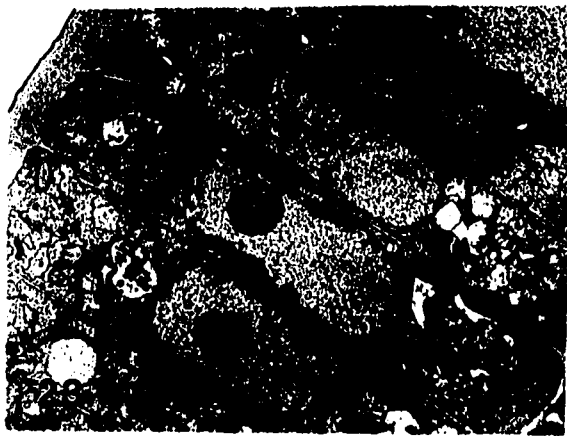
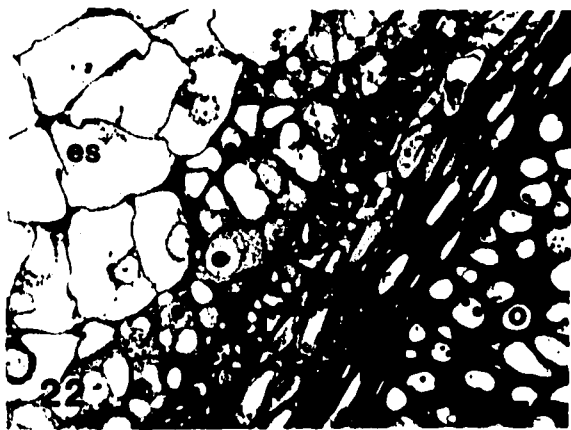
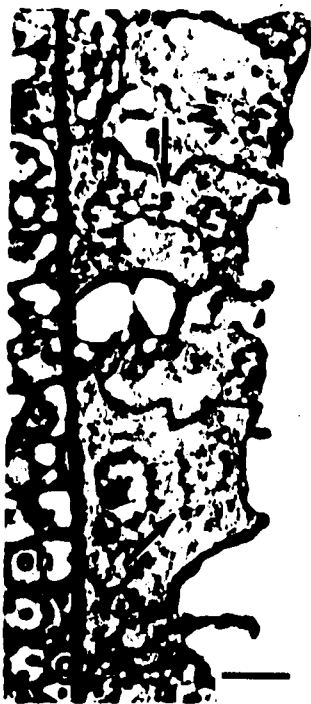
Figs. 12-17 Globular embryo stage. Fig. 12, anticlinal endosperm (es) wall ingrowth arising from the central cell wall showing accumulation of electron-dense material (arrow) in bulbous tip. c = central cell vacuole, bar = 0.2 μ m. Fig. 13, freely growing endosperm wall closely associated with dictyosome and vesicles. c = central cell vacuole; es = endosperm, bar = 0.2 μ m. Fig. 14, wandlabrinthe (w) with endosperm (es) wall ingrowth fused to base of suspensor (s) cell wall (arrow), bar = 1 μ m. Fig. 15, multilamellar body (*) in axil of anticlinal endosperm (es) wall ingrowth. en = endothelium, bar = 1 μ m. Fig. 16, sheet-like anticlinal endosperm (es) wall ingrowths protruding into central cell. Note vesicles near tips of developing walls (arrows). en = endothelium, bar = 10 μ m. Fig. 17, inner surface view of central cell showing cylinder-like ingrowths (*) of early cellularization of endosperm (es). en = endothelium, bar = 10 μ m.



Figs. 18-23 Globular (figs. 18-21) and early heart-shaped embryo (figs. 22, 23) stages.

Fig. 18, anticlinal endosperm wall ingrowths (arrows) and subsequent periclinal branching of these walls (arrowheads). en = endothelium, bar = 10 μ m. Fig. 19, phragmoplast formation (arrows) between dividing endosperm nuclei. en = endothelium, bar = 10 μ m.

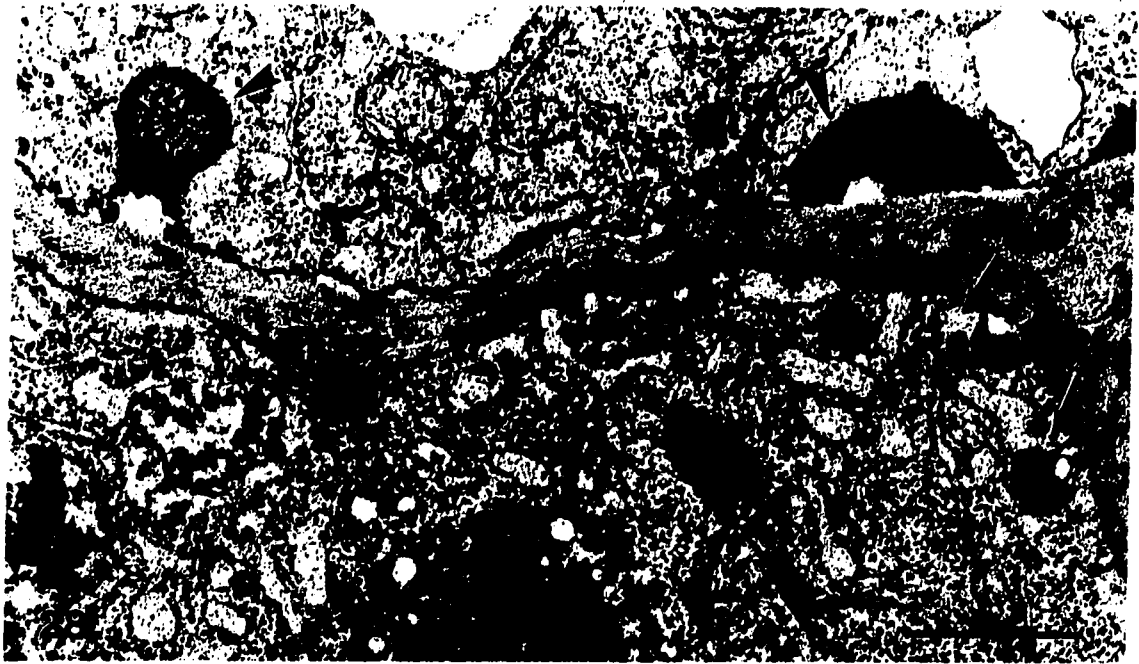
Fig. 20, endosperm (es) cellularization showing freely growing walls (arrows) and phragmoplast formation (arrowheads) after septation has occurred. en = endothelium, bar = 20 μ m. Fig. 21, chalazal process with free-nuclear endosperm (es) bordered by nucellar (n) cells laterally and hypostase (h) chalazally. i = inner integument; o = outer integument, bar = 20 μ m. Fig. 22, cross-section of ovular tissues showing cellular endosperm (es), different layers of inner integument (i), and inner-most layer of outer integument (o) infiltrated with dense mucilage around cells, bar = 20 μ m. Fig. 23, palisade epidermis with thick outer periclinal wall (arrow) showing cuticle, bar = 2 μ m.



Figs. 24-27 Late heart-shaped embryo stage. Fig. 24, embryo with dome-shaped apical shoot meristem (arrowhead) and procambial initials in hypocotyl (*). es = cellular endosperm; o = outer integument; s = suspensor, bar = 100 μ m. Fig. 25, horizontal-longitudinal section through ovule showing partially rotated embryo cotyledons (*) and cellular endosperm (es). Note constriction of embryo sac. o = outer integument, bar = 100 μ m. Fig. 26, cross-section of testa showing palisade epidermis (pa), hypodermis (hy), parenchymatous layer (*) and adjacent inner integument layer (far right), bar = 20 μ m. Fig. 27, tracheary bar (*) with pore opening to hilum (arrow). Note two-layered palisade epidermis (pa) in hilar region, bar = 100 μ m.



Figs. 28-30 Late heart-shaped embryo stage. Fig. 28, electron-dense material accumulated between plasmalemma and wall of endosperm cells in association with dilated rough endoplasmic reticulum (arrows). Plasmalemma invaginations (arrowheads) show regions where dense material will be released into cytoplasm where it diffuses (*), bar = 1 μ m. Fig. 29, chalazal process (cp) with endosperm cells embedded in a dense matrix. h = hypostase; i = inner integument, bar = 20 μ m. Fig. 30, granular matrix in chalazal process (cp). Note absence of central cell wall and persistence of the endothelium (en) cuticle lining chalazal process, bar = 1 μ m.



**PAPER 2: NUTRITION OF OVULE, EMBRYO SAC, AND YOUNG EMBRYO IN
SOYBEAN: AN ANATOMICAL AND AUTORADIOGRAPHIC STUDY**

**Nutrition of ovule, embryo sac, and young embryo in soybean: an anatomical and
autoradiographic study**

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ABSTRACT

Photosynthesizing soybean plants were exposed to $^{14}\text{CO}_2$ to study the incorporation of labeled water-insoluble photosynthates in ovules at various developmental stages. Using autoradiographic techniques on sectioned material, we show that the distribution of labeled carbon in different ovular tissues is regulated spatially and temporally. During zygote through globular stages of embryo development, labeled assimilates accumulate in integumentary tissue adjacent to the micropylar and chalazal poles of the embryo sac. A chalazal vascular trace and two adfunicular vascular strands are the pathways for accumulation of ^{14}C in these regions. Up through the proembryo stage, movement of labeled photo-assimilates into the lateral regions of the embryo sac seems blocked by a cuticle-like layer between the endothelium and embryo sac. At the globular embryo stage, the greatest accumulation of label is still at the chalazal and the micropylar ends of the embryo sac, but fragmentation of the cuticle-like barrier coincident with cellularization of endosperm allows channeling of labeled carbon from adjacent integumentary tissue into the embryo sac as well. Autoradiographic evidence for carbon flow into the embryo sac can be correlated with ultrastructural and morphological changes in time in ovular and endosperm tissues enclosing the embryo.

Key Words: autoradiography, embryo sac, Glycine, nutrition, ovule.

INTRODUCTION

Previous studies on legume *in vivo* embryo nutrition have speculated on the pathway of nutrient import (Hardham 1976; Thorne 1981; Offler and Patrick 1984; Folsom and Cass 1986). These studies concentrated on anatomical and cellular features of the ovule (distribution of plasmodesmata, wall-ingrowths, and vascular tissue) without confirmation of developmental data. Direct evidence for photosynthate uptake by legume seeds has been documented using $^{14}\text{CO}_2$, which is incorporated into photosynthates (Patrick and McDonald 1980; Thorne 1982; Grusak and Minchin 1988). Because the cotyledons are the principal storage organs of nonendospermic legumes at seed maturity, these latter studies concentrated on a stage during which the embryo was near maturity and the well-developed cotyledons were filling with reserves. No mention was made of earlier stages of ovule development. Moreover, because these studies were not coupled with autoradiographic evidence, only estimates of the pattern of photosynthate flux were made.

In this study, we concentrate on the nutrition of the early stages of embryo development in soybean. We provide autoradiographic evidence for water-insoluble ^{14}C -photosynthate flow into the embryo sac. This evidence is correlated directly with morphological and ultrastructural changes in ovular and endosperm tissues enclosing the embryo.

MATERIALS AND METHODS

Soybean plants, Glycine max (L.) Merr. cv. Harosoy, were grown either in a glasshouse or in the field. The pattern of floral development is the same for both locations. Floral buds or gynoecia isolated from flowers, at various stages of development, were placed in 3% glutaraldehyde - 4% paraformaldehyde in sodium cacodylate buffer (0.1M, pH 7.2) at room temperature (22 C, RT). The ovules were dissected in the fixative, placed under vacuum at 15 psi (6.89 kPa) for 1 h, and then placed in fresh fixative at 4 C for 12 h. Fixation was followed by three buffer rinses, postfixation in 1% osmium tetroxide (OsO₄) in the same buffer (pH 7.2) for 4 h at RT, and dehydration in a graded ethanol series. En bloc staining (5% uranyl acetate in 70% ethanol) was done during dehydration in some material processed for electron microscopy. Acetone was used as a transitional solvent prior to infiltration with Spurr's resin (hard recipe). Infiltration to pure resin was accomplished on a rotator over a two-week period (fresh resin daily) before embedding.

Light microscopy (LM)

Sections cut at 1 μ m thick with glass knives on a Reichert Ultracut E microtome were mounted on subbed (poly L-lysine or gelatin) glass slides. A double stain of 0.13% methylene blue - 0.02% azure II in 0.066M NaH₂PO₄ buffer (pH 6.9) followed by 0.2% basic fuchsin in 2.5% ethanol was used for its differential staining properties. All light microscope observations and photomicrographs were made with a Leitz Orthoplan microscope equipped with bright-field, differential interference-contrast (DIC), dark-field, and phase-contrast optics. Images were recorded on Kodak Technical Pan film.

¹⁴C-photosynthate labeling and autoradiography (AR)

The technique and apparatus for incorporation of labeled carbon into soybean ovules were based on those of Pleasants et al. (1990). Four glasshouse-grown flowering soybean plants were placed in a clear plexiglas chamber (0.6 x 0.6 x 0.9 m) located within an isotope-safe fume hood. The chamber was illuminated with two quartz lamps, which provided photosynthetically active radiation (PAR) of 100-150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The ¹⁴CO₂ was evolved by adding a drop (50 μl) of 6M HCl every 30 min to 1 mCi Ba¹⁴CO₃ (24.4 mCi/mmole) until all carbonate had reacted. The labeled carbon dioxide was circulated continuously through the sealed chamber by an aquarium pump for an incorporation period of 6 h. The unincorporated ¹⁴CO₂ was purged from the system by rerouting gas circulation through saturated KOH. Subsequently, plants were removed to the glasshouse.

Gynoecia and floral buds were removed at regular intervals (0-25 d) postincorporation and at various stages of postfertilization development. The material was fixed, dehydrated, infiltrated, and embedded as described. Thick (1 μm) resin sections were placed on poly L-lysine subbed glass slides and left unstained. A wire loop was used to apply a thin, even coat of liquid autoradiographic emulsion (Kodak NTB2). Slides were allowed to dry for 30 min at 45 C, put into sealed light-tight slide boxes with a desiccant (CaSO₄), and placed in the refrigerator (5 C) for 7 d. After this exposure period, slides were removed, allowed 1 h to come to RT, and processed. For complete procedures for emulsion handling, slide preparation, and processing, see Technical Notes P-64, Eastman Kodak Company, Health Sciences Division, Rochester, New York. After processing of the autoradiographic emulsion, slides were dehydrated in a graded ethanol series followed by xylene. The sections were covered with Permount and coverslipped. The emulsion-covered sections were observed and photographed.

Image analysis

DIC images were digitized with the Leitz Orthoplan fitted with a video camera (COHU model 4815-5000) connected to a video digitizer (Colorado Video Inc. model 270A). The digitized images were saved and manipulated with a Kevex Delta IV system and a Kevex Feature Analysis software program. This image analysis system allowed the silver grains in the emulsion over the sections to be quantified. These grains were indicative of incorporated water-insoluble ^{14}C photosynthate. Twenty discrete tissues or regions of the ovular sections (ex: micropylar outer integument, suspensor, and endothelium) were analyzed for the abundance of silver grains, to determine the pattern of ^{14}C incorporation. Different areas within a given region or tissue were sampled from numerous sections of various ovules (12-15 ovules per stage of development). The silver grains were enhanced and the pixel areas covered by the grains recorded via image analysis. Numbers of silver grains were estimated from the pixel area coverage; background grains were subtracted; and the number of grains averaged per unit area ($1000\ \mu\text{m}^2$) for each region of the ovule at each stage of development. Diagrams of three of the five stages of ovule development summarize the average number of silver grains by regions in the ovule (see Figs. 7, 18, 30).

Transmission electron microscopy (TEM)

Thin sections (60-90 nm) were cut using a Diatome diamond knife and stained with 5% uranyl acetate in 70% ethanol for 1 h, and in lead citrate for 1 h. Sections were observed and photographed on a Hitachi HU-11C-1 TEM at 75 kV or a Jeol JEM-1200EX-II STEM at 80 kV.

RESULTS

The ovule of soybean is bitegmic, crassinucellate, and campylotropous. In median longitudinal sections of the ovule (Fig. 1), this latter feature is most evident. During megasporogenesis, each ovule (usually three per ovary) bends towards the distal end of the gynoeceium, the outer integument becoming adnate to the funiculus for part of its length. This ventral portion of the ovule facing the placenta and funiculus, and the opposite tissues are referred to in this study as the adfunicular and abfunicular portions of the ovule, respectively.

Zygote stage

Preceding and immediately after fertilization, the central cell of the megagametophyte is filled with numerous osmiophilic bodies and large amyloplasts (Fig. 2). Curiously, the central cell starch reserves are labeled rarely (Figs. 3, 4). These amyloplasts are only labeled in ovules initiating development more than 12 days postexposure to $^{14}\text{CO}_2$. This implies that they are not formed from photo-assimilates directly, but a remobilized unlabeled carbon source.

Divisions of the endosperm precede those of the zygote. Coincident with the initial free-nuclear divisions of the endosperm, the central cell starch reserves diminish. Unlike the large amyloplasts, the thin cytoplasm of the central cell has noticeable label. The organellar complement of the central cell is more concentrated at its micropylar end, near the zygote and the wandlabrinthe.

The zygote shows a distinct polarity of its cytoplasm: two pockets of cytoplasm are separated by a large central vacuole. The micropylar cytoplasm of the zygote is almost completely devoid of storage material, but the chalazal-most cytoplasm, containing the

nucleus, is rich in osmiophilic bodies and amyloplasts (Fig. 2). The zygote lacks a cell wall, and it is connected to the central cell by plasmodesmata, suggesting that the zygote has symplastic contact with it.

The wandlabrinthe, as described by Tilton et al. (1983, 1984), is a localized proliferation of cell wall material projecting from the micropylar central cell wall towards the zygote. In the present study, the wall ingrowths extend to the height of the zygote along the adfunicular central cell wall, but are limited to a third of its height on the abfunicular side. The preferential proliferation of the wandlabrinthe along the adfunicular side of the central cell is consistent through the late heart-shaped embryo stage.

Authoradiographic evidence suggests that the wandlabrinthe is involved directly with the transport of labeled assimilates into the embryo sac. The micropylar base of the embryo sac and the facing integuments are labeled heavily (Fig. 3). Label is concentrated over the wandlabrinthe, suggesting a pathway of solute transport to the base of the zygote and central cell from the outer integuments. Supporting this route of nutrient flow, label is present in the cytoplasm of the embryo and in the thin layer of cytoplasm encircling the embryo and the primary endosperm nucleus (Fig. 3).

The nucellus enclosing the conical embryo sac is composed of large, highly vacuolated cells generally parallel to the longitudinal plane of the embryo sac (Fig. 4). Their cytoplasm contains amyloplasts, RER, free ribosomes, and dictyosomes. At this stage of development, plasmodesmata connect adjacent nucellar cells, but no connection is observed with the central cell, zygote, degenerating synergids, or integuments. Nucellar cells with wall ingrowths (transfer cells) are infrequent, and ingrowths are limited to a few small protrusions per cell. Nucellar cells along the inner integument lack wall ingrowths. Frequently, a single plasmodesm traverses a wall ingrowth and, moreover, may connect it

with a second ingrowth in an adjacent cell (Fig. 5). In general, the nucellus is weakly labeled (Fig. 3, 4).

In median longitudinal sections, the nucellus and embryo sac are flanked by the inner integuments, at the micropylar end by the outer integument (Fig. 3), and at the chalazal end by the hypostase (Figs. 4, 6). The interface between the inner integument and nucellus is marked by a cuticle-like layer which is conspicuously absent at the micropylar and chalazal ends of the nucellus (Figs. 3, 4). This cuticle forms a cylinder around the embryo sac and nucellus which is only open at the chalazal and micropylar ends to the outer integuments. This dense osmiophilic layer is deposited by the inner integument on its inner surface as it encloses the nucellar tissue during megagametogenesis (unpublished data). This cuticle is resistant to cellulase and pectinase digestion (Chamberlin et al. in press). Enzyme digestion of ovules for 12 h yielded embryo sacs completely devoid of integuments, exposing the cuticle-like layer. Embryo sacs remained intact with no apparent digestion of the cuticle-like layer after 48 h in the enzyme solution. No histochemical evidence is given here to verify the nature of this cuticle-like layer. It appears to be the same as the electron-dense material that covers the epidermis of the ovule; both are assumed to be cuticles. The pattern of ^{14}C label adjacent to the cuticle indicates that this layer is a barrier to solute movement. Labeled carbon accumulates in the inner integuments, but only small amounts of label occur in the adjacent nucellus (Fig. 3).

The hypostase tissue at the chalazal end of the nucellus is composed of a small group of thick-walled cells bound laterally by the cuticularized inner integument (Fig. 4). These cells are densely cytoplasmic and rich in organelles, including amyloplasts, and have numerous vesicles that suggest high metabolic activity. The chalazal-most cells of this grouping often possess thin end walls fronting the terminus of the chalazal vascular strand. This condition is mirrored in the more micropylar cells of the hypostase facing the nucellus.

Numerous plasmodesmata connect these cells longitudinally. Labeled assimilates are concentrated heavily in the hypostase and to a lesser extent in the adjoining tissues (Fig. 4). The chalazal end of the central cell shows distinct labeling as well. The outer integumentary cells, chalazal to the hypostase, show a diffuse pattern of labeling concentrated around the vascular strand. A summary of incorporated label in the ovule, by region, is shown in Fig. 7.

The outer integument is variable in thickness (3-15 cell layers) and adnate to the inner integument. The inner integument is 2-3 cell layers thick and has a distinct cuticle on its inner surface opposing the nucellus (Figs. 3, 4). The nucellus is enclosed incompletely by the inner integument and its micropylar and chalazal ends are exposed to the outer integument. No plasmodesmata connect the cells of the inner integument with those of the outer integument (Fig. 8) although plasmodesmatal connections are common between cells within each integument. This symplastic isolation of the two integumentary layers is observed in the later stages of ovule development as well. In general, the integumentary cells are rich in RER, ribosomes, dictyosomes, and mitochondria. Cells with starch-filled plastids are notably abundant in the: 1) inner portion of the outer integument along the entire adfunicular side; 2) micropylar abfunicular outer integument; 3) micropylar end of the inner integument; and 4) funiculus. The ^{14}C labeling is consistent with the aforementioned pattern of starch accumulation (Fig. 7). This pattern in the integuments reflects a pathway of assimilate transport to the embryo sac. The adfunicular outer integument shows a 2-3 fold greater concentration of label than does its abfunicular counterpart.

A single unbranched vascular strand originating from the funiculus enters the ovule via the chalazal end of the outer integument. This collateral bundle, embedded 2-3 layers below the epidermis of the ovule, terminates just chalazal to the hypostase (Fig. 6). Within

the bundle, the sieve elements occupy the chalazal-most portion of the vascular strand. Although the vascular strand proper is not labeled heavily, the adjacent parenchymatous cells show elevated levels of incorporated ^{14}C . Because only the funiculus connects the ovule to the gynoecium, and therefore to the parent plant, this vascular bundle plays a primary role in the nutrition of the ovule.

Proembryo stage

The zygote begins to divide 2-3 d after fertilization, forming the proembryo, a two- to multicellular embryo not yet differentiated into embryo proper and suspensor. The basal-most cells of the proembryo are highly vacuolate (Figs. 9, 10) whereas the apical cell and its immediate neighbors are densely cytoplasmic (Fig. 9). The large amyloplasts and osmiophilic bodies, prevalent in light micrographs of the zygote, are now absent, replaced by numerous plastids without starch in the apical cells of the proembryo. Wall ingrowths develop from the tangential and basal cross walls of the basal-most cells of the proembryo (Fig. 10). As the embryo divides, the frequency of wall ingrowths in these cells increases. The cells of the proembryo are interconnected by plasmodesmata. The wandlabrinthe arising from the central cell wall, extends from the basal cell of the proembryo (Fig. 10); it has proliferated from the zygote stage. Autoradiographs demonstrate the importance of the proembryo wall ingrowths and of the wandlabrinthe. Label is concentrated in the cell walls of the proembryo and particularly in the basal cell (Fig. 9). The wandlabrinthe also is labeled heavily.

The most obvious change within the central cell at this stage of development is the expansion of a large central vacuole displacing the free-nuclear endosperm and starch aggregates to the periphery. This central vacuole concentrates no label. The divisions of the free-nuclear endosperm outpace cell proliferation in the proembryo. Concomitant with

the increase in free-nuclear endosperm is the rapid decline in central cell starch reserves. The loss of starch aggregates is complete when the embryo is 16-celled (5-6 d postfertilization).

As the central cell expands, the nucellus degenerates. Nucellar cells show signs of breakdown well before physical contact with the central cell. The walls of the nucellar cells become progressively thinner as mucilage-like material occurs between the walls and plasmalemmas (Fig. 11). This material eventually fills the nucellar cells before they rupture. The electron-dense material does not accumulate to any extent in the central cell and, therefore, may be absorbed by the central cell in some other form. As the nucellus diminishes, the enlarged embryo sac is exposed laterally to the inner integuments, and at the micropylar end to the outer integument.

The chalazal end of the central cell, its adjacent nucellus, and the hypostase remain heavily labeled, similar to that at the zygote stage. The outer integumentary cells opposite the hypostase continue to concentrate label, as does the funiculus, which supports this region with a vascular strand. The ovule rapidly expands. A large portion of the starch accumulated in the integuments at the zygote stage is now depleted. The most significant accumulation of starch and label are in the chalazal and the micropylar ends of the integuments opposite the embryo sac. There is sparse label in a band of parenchyma cells embedded in the adfunicular outer integument.

Globular embryo stage

The embryo differentiates into a globular head (embryo proper) and a suspensor approximately 8 d postfertilization (Fig. 12). No wall ingrowths are seen in the cells of the embryo head (Fig. 13). The cells of the globular head and suspensor are interconnected via plasmodesmata as are the basal-most cells of the suspensor and the surrounding central cell.

All suspensor cells have wall ingrowths, their abundance per cell decreases acropetally within the suspensor (Fig. 14). Immediately before the cotyledons are initiated, a thin cuticle forms over the surface of the globular head but this cuticle is absent around the suspensor.

In autoradiographs, label continues to be concentrated at the micropylar end of the embryo sac (Fig. 12). The basal-most cells of the suspensor are heavily labeled, with the label decreasing apically to the globular head. The cellular endosperm bordering the wandlabrinthe also shows significant label. The central cell vacuole, partly surrounding the embryo, has little to no label (Fig. 15).

As the ovule enlarges longitudinally, thin regions and then gaps develop in the cuticle-like layer separating the inner integument from the nucellus or the endosperm (Figs. 16, 17). The formation of these cuticular breaks coincides with and follows the same pattern as cellularization of the endosperm which occur first at the micropylar region of the central cell and later at the chalazal end. Often these cuticular breaks appear immediately before and opposite the peg-like wall ingrowths of the central cell wall (Fig. 16). With the formation of the cuticular gaps, a decreasing gradient of label is now observed from the inner integument to the cellular endosperm and central vacuole (Fig. 15). The label is concentrated in or along the walls of the cellular endosperm and not in its cytoplasm. The pattern of ^{14}C label in the ovule is illustrated (Fig. 18).

The inner integument differentiates into an inner layer of endothelial cells (integumentary tapetum) and outer layers (1-7) of thick-walled cells (Fig. 19). The thick-walled cells appear metabolically inactive (i.e., a single large vacuole displaces a thin layer of cytoplasm, which is sparse in organelles). The endothelial cells which border the endosperm are densely cytoplasmic and rich in ribosomes, vesicles, mitochondria, RER, dictyosomes, and plastids. Plasmodesmata are common in the endothelial cell walls. Few

plasmodesmata exist between the endothelium and the outer layers of the inner integument, and these are obliterated as the walls of the latter thicken. No plasmodesmatal connections exist between the inner integument and the embryo sac. All cell layers of the inner integument are labeled equally (Fig. 15).

The chalazal end of the embryo sac becomes distended as the ovule expands, forming an elongate channel to the hypostase (Fig. 1). This channel is bound laterally by undigested nucellar tissue and is filled with free-nuclear endosperm embedded in dense cytoplasm (Fig. 20). Label is heavily concentrated in the endosperm of the chalazal channel and to a lesser extent in the adjacent nucellus (Figs. 18, 20). The immediate source of label for these tissues seems to be the hypostase positioned chalazal to the channel.

The funiculus is heavily labeled along its length in the region of the single vascular bundle (Fig. 21). As the funicular bundle enters the ovule it branches, and these branches are clearly depicted by three paths of label. One path of the label follows the vascular bundle into the chalazal outer integuments terminating opposite the hypostase. The parenchymatous cells surrounding the trace are densely cytoplasmic (Fig. 22) with abundant plasmodesmata. The majority of label is localized just chalazal to the sieve elements in a layer of parenchymatous cells enriched with osmiophilic bodies and starch (Fig. 23). The second and the third paths of label, only one of which can be seen per median longitudinal section, pass through the entire length of the adfunicular outer integument (Fig. 21). These latter branches are two equal and parallel strands of provascular tissue, which branch from the funicular bundle as it enters the ovule (Fig. 1). The provascular cells are densely cytoplasmic and have numerous plasmodesmata. The terminal portion of each provascular strand ends in the outer integument opposite the micropylar end of the embryo sac. At the terminus of each of these two bundles is a single group of cells with large aggregates of osmiophilic bodies of differing shapes and numerous amyloplasts (Figs. 24, 25). Among the

ovular tissues, these cells have the most pronounced accumulation of label observed in this study (Figs. 12, 18).

Early heart-shaped embryo stage

The embryo expands differentially to form the two cotyledonary primordia 12-15 d postfertilization (Fig. 26). As in earlier stages of development, the cells of the embryo proper and suspensor are interconnected by plasmodesmata, but no such connections exist with the surrounding endosperm at this and at subsequent stages. The suspensor is now elongated and consists of approximately 10 tiers of isodiametric cells. The basal three tiers of suspensor cells contain wall ingrowths of the pattern mentioned previously. Vesicles are especially numerous along the plasmalemma of the transverse walls of the suspensor cells. Plasmodesmata are more abundant in the transverse walls of the suspensor cells as well. There is a decreasing amount of label from the base of the suspensor to the embryo proper (Fig. 27). The thin cuticle formed at the late globular embryo stage remains conspicuous over the surface of the embryo proper during this time and later stages.

The wandlabrinthe is distinct and extends to the height of the fourth tier of suspensor cells along the adfunicular central cell wall (Fig. 28). The abfunicular portion of the wandlabrinthe is not as extended. During cellularization of the endosperm, some anticlinal walls arising from the wandlabrinthe are fused to the exterior walls of the suspensor. This contact confers direct apoplastic continuity between the wandlabrinthe and embryo. At this stage, as in earlier stages, the wandlabrinthe is heavily labeled.

Except for the chalazal channel, the endosperm is completely cellular. The central endosperm is composed of large thin-walled cells, each with a single large vacuole, but no visible storage material (Fig. 26). Plasmodesmata are rare in the central endosperm cells, in contrast to the peripheral layer. The central endosperm is weakly labeled. The free-

nuclear endosperm of the chalazal channel has enlarged nuclei embedded in cytoplasm poor in organelles. As in earlier stages, this channel is heavily labeled.

The outer integuments undergo morphological changes important to nutrient flux during the early heart-shaped embryo stage. Two distinct vascular bundles differentiate from the provascular traces in the adfunicular outer integument. Connected to the two vascular bundles, provascular tissue in the lateral regions of the outer integuments differentiates into numerous parallel minor veins. These minor veins branch and anastomose to form a network extending to the abfunicular side of the ovule (Fig. 29). The minor veins consist mainly of sieve elements. Coincident with the vascularization of the whole ovule, label is found abundantly throughout the integumentary tissues. The concentration of label, by region, in the ovule is diagrammatically represented in Figure 30. In earlier stages, only the adfunicular integuments showed substantial label (Figs. 7, 18).

Although the integuments generally are well labeled, the greatest concentrations of label are in tissues associated with the principal venation of the ovule. Parenchymatous cells adjacent to the two adfunicular bundles are rich in starch and osmiophilic bodies. At the ends of these two bundles, there persists a group of cells just opposite the micropylar end of the embryo sac that has the greatest concentration of label in the ovule (Fig. 27, 30). A similar, but less extensive, reservoir of storage materials exists immediately chalazal to the chalazal vascular strand, as already shown for the globular embryo stage (Fig. 23). The micropylar adfunicular inner integument is also heavily labeled, even though it is not close to the vasculature nor does it have the obvious storage reserves of the aforementioned tissues.

As label accumulates in the integuments, there is a concomitant increase in its concentration in the endosperm (compare Figs. 18 and 30). The cuticle separating the embryo sac from the inner integument is fragmented and only remnants remain. In the

absence of this cuticle, a decreasing concentration gradient of label exists from the inner integument to the central endosperm cells. This pattern of label from the inner integuments to the endosperm does not include the embryo proper. In autoradiographs, the endosperm surrounding the embryo proper is labeled only slightly, but the embryo has an eleven-fold greater accumulation of label (Fig. 30). This indicates that the embryo proper accumulates label via the suspensor.

Late heart-shaped embryo stage

Approximately 25 d postfertilization, the embryo has become structurally mature. Apical and radicle meristems have differentiated from the embryo proper. The bulk of the embryo consists of the two cotyledons and occupies the micropylar half of the embryo sac. Provascular strands in the cotyledons branch and anastomose to form a network of vasculature. The cotyledons begin to accumulate starch, lipids, and proteins. Although the cotyledons are accumulating storage reserves, they are not strongly labeled.

The suspensor has doubled in length due to elongation of its cells (Fig. 31). The organellar complement of the suspensor cells indicates high metabolic activity. Plasmodesmata are common in the walls interconnecting suspensor cells. The basal-most tier of suspensor cells continues to display wall ingrowths, as in earlier stages. These wall proliferations are on the tangential walls in contact with the endosperm and the basal transverse walls bordering the central cell wall. A cuticle, prominent on the surface of the embryo (Fig. 32), is absent on this basal-most tier of suspensor cells, intimating the importance of these cells to nutrient absorption. Autoradiographs indicate that the label is concentrated in the base of the suspensor, and in the endosperm and wandlabrinthe surrounding it.

Cellularization of the endosperm is complete, including the chalazal process (Fig. 33). This process and the proximal hypostase are as heavily labeled as in earlier stages. The outermost layers of endosperm cells continue to divide as the embryo sac expands. The cellular endosperm shows signs of degeneration during the expansion of the embryo into the embryo sac. Loss of endosperm is not due solely to the encroachment of the embryo. Some endosperm cells degenerate although they are not in physical contact with the embryo (Fig. 31). These degenerating cells have numerous vesicles fused to their plasmalemmas (Fig. 34). A loose network of individual fibrils are observed at the degenerating endosperm walls. An electron-dense, mucilage-like material forms in the voids left by these degenerating cells. This electron-dense material is composed of the fibrillar material (Fig. 34).

As the ovule continues to expand, the outer layers of thick-walled cells of the inner integument become progressively thinner, until only wall remnants remain. In contrast, cell divisions of the endothelium keep pace with the expansion of the ovule and this layer remains distinct until seed maturity. Degradation of the cuticle, which separates the embryo sac from the endothelial layer, is complete (Fig. 35). The gradient pattern of label from the integuments to the peripheral endosperm layers is consistent with the early heart-shaped embryo stage. The central endosperm cells are highly vacuolate and lack storage materials. These cells are weakly labeled in comparison to the embryo and integuments.

Embedded within the parenchyma of the outer integument are the two parallel adfunicular vascular strands, each terminating near the micropylar end of the embryo sac. Cells along and near the terminus of these vascular strands continue to contain many osmiophilic bodies and starch. The existing minor vascular branches, arising from the two strands, are more extensive and accommodate the enlargement of the ovule. The chalazal vascular strand remains unbranched but labeled. The paravascular cells in this region

contain many osmiophilic bodies and starch. The general pattern of carbon labeling in the integuments remains unchanged from the early heart-shaped embryo stage (Fig. 30).

DISCUSSION

$^{14}\text{CO}_2$ coupled with autoradiography is useful in following the pattern of photosynthate incorporation and transport in developing ovules and its ultimate accumulation in the embryo. This study shows that the nutrition of the soybean ovule is spatially and temporally regulated. In the initial zygote and proembryo stages of development, nutrients appear to enter the micropylar and chalazal poles of the embryo sac via the wandlabrinthe and hypostase, respectively. This bipolar pathway of nutrient flux is likely dictated by the presence of the cuticle-like barrier at the interface of the inner integument and embryo sac. At the globular embryo stage, this barrier degenerates and nutrients appear to flow relatively unimpeded into the embryo sac laterally to supplement the nutrient flux at the two ovular poles. In subsequent stages, the complete vascularization of the outer integument accommodates the passage of nutrients over the entire surface of the embryo sac. As discussed, numerous tissues are directly and indirectly involved in the nutrition of early embryogenesis in soybean.

The wandlabrinthe, acting as a nutrient channel, has been implicated as the "source" of carbohydrates for the synthesis of the starch packets in the central cell (Folsom and Cass 1986). This contradicts the observations of Kennell and Horner (1985) who showed that these reserves are formed early, during the megagametophyte stage, and that one-third to one-half of the starch in the soybean central cell had disappeared by the time of wandlabrinthe formation. In autoradiographs, we observed that the central cell starch aggregates were not labeled. This absence of label suggests that the immediate source of carbohydrates for this starch is an unlabeled one. This unlabeled source would seemingly exclude the wandlabrinthe and hypostase, which are heavily labeled channels of

photoassimilate flux. The nucellus is weakly labeled with ^{14}C and its breakdown products may be a source of materials for the formation of starch grains in the embryo sac.

Breakdown of both the unlabeled starch packets and the nucellus could serve as a pool of nutrients in the central cell, which initially would be available to the embryo and endosperm. Because endosperm nuclear divisions precede cell divisions of the embryo it is likely that the free-nuclear endosperm is the recipient of a major portion of these central cell reserves. The central cell is connected via plasmodesmata with the zygote, proembryo (Dute et al. 1989), and early globular embryo (present study). This cytoplasmic continuity indicates that the reserves of the central cell are readily accessible to the zygote/embryo.

The initiation of transfer cell-like wall ingrowths at the transverse and tangential walls of the basal cells of the proembryo suggests that these cells are now actively involved in the absorption of nutrients and this is supported by autoradiographic evidence. Label is associated with the wandlabrinthe and its adjoining cytoplasm near the base of the proembryo, and then it occurs acropetally within the embryo. Due to the absence of a cuticle, the proembryo also may absorb the reserves of the central cell over its entire surface. Whether the embryo receives nutrients from the central cell remains unconfirmed. Because the starch packets are unlabeled and the nucellus is only weakly labeled, the assimilation of their breakdown products into the embryo could not be determined.

Through the early globular embryo stage, integumentary storage reserves move into the embryo sac only through the chalazal and micropylar ends as indicated by the concentration of label in these regions. These two pathways for labeled assimilates are a consequence of a lateral barrier, the continuous, dense, osmiophilic, cuticle-like layer between the endothelium and embryo sac. This electron-dense band does not extend beyond the limits of the inner integument, therefore, the embryo sac and nucellus are enclosed only laterally. We found that the layer is impervious to enzymatic digestion by cellulase and pectinase.

Similar results, including histochemical and acetolysis experiments, indicate that a similar endothelial band in Antirrhinum ovules is composed of highly stable lipid substances, probably cutin or sporopollenin (Yang 1989). Endothelial cuticles have been observed in other genera, Phaseolus (Offler and Patrick 1984) and Saintpaulia (Mogensen 1981). The embryo sac is isolated by the endothelial cuticle at a time when its nutrient requirements are fulfilled from within by starch reserves or by transport of solutes through the poles of the embryo sac. This cuticle-like layer may be retained during the early stages of embryo sac development to inhibit the outflow of solutes from the nutrient rich embryo sac to the integuments. As development of the globular embryo and endosperm proceeds, these reserves are rapidly depleted and thus the requirement for increased nutrient flux from outside the embryo sac is enhanced. The lateral integuments are, therefore, exposed to the embryo sac during the degeneration of this cuticle-like barrier to allow movement of labeled assimilates from the integuments to the embryo sac. The gaps in the cuticle-like layer are likely formed by the physical stretching of this layer during the longitudinal expansion of the embryo sac.

At the micropylar end, the wandlabrinthe is the site of assimilate movement into the embryo sac. This labyrinth of wall ingrowths is a common occurrence in many angiosperm genera including the legumes Pisum (Hardham 1976), Vicia, Phaseolus, Spartium, and Lathyrus (Gunning and Pate 1974). As in soybean (Tilton et al. 1984), the wandlabrinthe of these latter genera is purported to facilitate the transport of solutes to the embryo sac.

The wall elaborations of the wandlabrinthe likely have an analogous function to those in transfer cells. Wall ingrowths of transfer cells facilitate solute uptake by their heightened plasmalemma surface area (Wimmers and Turgeon 1991). The number of potential sites at which enzymatic reactions could occur would be increased by heightened plasmalemma surface area. An active transport mechanism has been shown by Brentwood and Cronshaw

(1978) who correlated ATPase activity and the formation of wall ingrowths in Pisum phloem transfer cells. ATPase activity has also been localized at the central cell plasmalemma of Helianthus (He and Yang 1991a) and Antirrhinum (He and Yang 1991b). These authors ascribed a nutrient absorbing function to the enzyme in the central cell.

Autoradiographic evidence implicates the hypostase as the second channel of nutrient movement into the embryo sac. Its position in the pathway of the chalazal vascular bundle and its anatomical features make it a logical choice for this function. The hypostase cells are densely cytoplasmic and rich in organelles, and its thickened walls with numerous plasmodesmata indicate an active tissue probably involved in symplastic transport. The presence of ATPase activity in the hypostase cells of Antirrhinum ovules also implies an active transport function to this tissue (He and Yang 1991b). Its function in the translocation of nutrients to the embryo sac also has been suggested in Ornithogalum (Tilton 1980). Contrary results were found in a $^{14}\text{CO}_2$ autoradiographic study in Zephvranthes (Coe 1954). Although the chalazal portion of the ovule, vascular tissue, and embryo sac were heavily labeled, the hypostase was not labeled. Coe concluded that the hypostase is an impediment to nutrient flux and that ^{14}C assimilates circumvent the hypostase and enter the embryo sac laterally. The hypostase occurs widely in a number of taxa of diverse families as noted by Maheshwari (1950) and Tilton and Lersten (1982).

At the zygote through globular embryo stages, no vascular tissues connect the funiculus to the micropylar end of the integuments, in contrast to that at the chalazal end. Therefore, the abundance of nutrients in the micropylar adfunicular integuments are not easily explained. Autoradiographic evidence indicates that solutes are transported to the micropylar integuments through a band of densely cytoplasmic parenchymatous cells embedded within the adfunicular outer integument. This zone of cells abuts the single vascular bundle entering the ovule from the funiculus and extends to the micropylar end of

the ovule opposite the embryo sac. Transport is most likely symplastic down a steep concentration gradient created by the unloading of solutes from the funicular vascular strand to the adfunicular outer integument and their assimilation at the micropylar end of the embryo sac.

At the globular embryo stage, the tissue in this region will eventually give rise to the two adfunicular provascular strands. It may be assumed, therefore, that these cells are already genetically programmed to function in the transport of solutes to the micropylar integuments as they develop into distinct vascular bundles. Label appears to be translocated within these bundles and unloaded at their termini in the micropylar outer integument. The label is then localized in a group of cells forming a rich reservoir of labeled starch and dense material opposite the micropylar end of the embryo sac. These reserves move to the embryo sac and are continually replenished with a labeled carbon source, as suggested by autoradiographic evidence. Along the length of the adfunicular bundles, label passes from the outer integument through the inner integument to enter the lateral regions of the embryo sac. The pattern of label suggests that nutrients pass from the inner integuments into the lateral portions of the embryo sac. The anticlinal wall ingrowths formed during cellularization of the endosperm probably function analogous to those in transfer cells, increasing the surface area of the central cell to enhance nutrient transport into the embryo sac.

During the late globular stage, the embryo forms a cuticle over its surface, but it is notably absent from the suspensor. Although the cuticle of the embryo is not as distinct as that of the inner integument, it may be impervious to solute exchange. The cuticularization of the embryo proper and its lack of contact with the heavily labeled outer endosperm layers suggest that the embryo proper, and later, the young cotyledons are nonabsorptive. The embryo likely receives the bulk of its nutrition at the micropylar end of the embryo

sac via the suspensor. The potential for nutrient absorption and transport by the embryo is increased by the proliferation of cell wall ingrowths in the suspensor cells. The presence of suspensor wall ingrowths and its function in the absorption and transport of nutrients has been proposed for some taxa: *Phaseolus* (Schnepf and Nagl 1970; Yeung and Clutter 1978), *Capsella* (Schulz and Jensen 1969), *Pisum* (Marinos 1970), *Epilobium*, *Antirrhinum*, *Vicia*, *Lathyrus*, and *Scrophularia* (Gunning and Pate 1974). In soybean, a concentration gradient of label within the suspensor indicates that the basal-most cells absorb nutrients from the embryo sac and/or wandlabrinthe which are then transported acropetally to the embryo proper. During early embryogenesis of *Phaseolus*, Yeung (1980) found that labeled sucrose was preferentially absorbed by the embryo via the suspensor, suggesting that the suspensor functions in the nutrition of the embryo.

Our study shows that the soybean chalazal process has a role in the transport of nutrients into the embryo sac. This is supported by the concentration of label in the chalazal free-nuclear endosperm and the position of the process proximal to the labeled chalazal vascular strand. Label accumulates in the chalazal process from the hypostase and from the lateral integuments via the remnant of the nucellus. The chalazal process remains free-nuclear until the late heart-shaped embryo stage. This delayed cellularization indicates that maintenance of a symplastic channel is important to its function in nutrient absorption and transport. As cellularization of the endosperm proceeds, the chalazal process seems to become progressively isolated from the rest of the embryo sac. Although its free-nuclear endosperm continues to concentrate labeled materials, they are probably utilized in the subsequent cellularization of this region, or in limited transport to the proximal parts of the embryo sac.

The early heart-shaped embryo stage is marked by the vascularization of the outer integuments. Autoradiographic evidence indicates that the bulk of the label moves from

these vascular strands towards the embryo sac. The unloading of solutes from the vasculature of the soybean ovule is a metabolic process facilitated by transfer cell-like parenchyma cells in intimate contact with the sieve elements (Thorne 1981, 1982). The path of nutrient flux from these parenchymatous cells to the endothelium is likely apoplastic, because of the absence of plasmodesmatal connections between the inner and outer integuments. The transport force for this nutrient movement may be diffusional along a concentration gradient created by the unloading of photosynthates at the vasculature and their assimilation in the embryo sac. This is supported by the gradient pattern of label in these tissues. Thorne (1980) observed a lag period between the incorporation of ^{14}C in the outer integument and its accumulation in the embryo. The author concluded that this lag period was due to passive diffusion of ^{14}C -photosynthates from the vascular tissue of the outer seed coat to the inner integuments. Our observations confirm those of Thorne (1980).

The differentiation of the inner integument into two morphologically distinct layers at the globular embryo stage suggests a division in function as well. The cells of the outer layer, with their reduced organellar complement and thickened walls lacking plasmodesmata, probably have a passive role in nutrient flux. The endothelial cells are densely cytoplasmic and rich in organelles and have numerous plasmodesmata. The endothelium with its cytological features seems to be well adapted for active transport of nutrients to the embryo sac. The pattern of ^{14}C indicates that nutrient flux is unimpeded from the integuments to the endosperm, at the onset of degeneration of the cuticle-like layer of the endothelium. This mobilization of nutrients to the endosperm is likely an energy-requiring process mediated by the hydrolysis of ATP as is suggested by ATPase activity at the plasmalemma of endothelial cells of *Saintpaulia* (Mogensen 1981), *Antirrhinum* (He and Yang 1991a) and *Helianthus* (He and Yang 1991b). The endothelium

may also actively accumulate nutrients against a concentration gradient, as suggested by the high concentration of label in the micropylar adfunicular inner integument in this study.

Label accumulates principally in the peripheral layers of endosperm and is likely used as a substrate for the continued divisions of the endosperm. As cellularization of the endosperm is completed, except for the chalazal process, label does not appear to move from the peripheral layers to the centripetal endosperm cells. Because of the thin walls, paucity of organelles and plasmodesmata, and the absence of storage products, the centripetal endosperm is likely to be insignificant in the nutrition of the encroaching embryo. Although, poorly labeled, the cellular endosperm may store water-soluble nutrients originally stored in the large central vacuole of earlier stages. These water-soluble substances would have been lost during chemical fixation and dehydration and, therefore, the tissues would be weakly labeled. Freeze substitution methods coupled with autoradiography in a study similar to the present one is needed to determine if the central vacuole or central endosperm store these water-soluble nutrients.

The cellular endosperm may act as a limited nutrient source for the developing embryo. The fibrillar material, which appears during the degeneration of the endosperm walls, seems to coalesce to form an electron-dense material between the endosperm cells. It is possible that these fibrils are cellulosic wall material, and the electron-dense material is a converted form of carbohydrate that is absorbed by the embryo. The evident lack of additive accumulation of this substance in the embryo sac suggests this interpretation.

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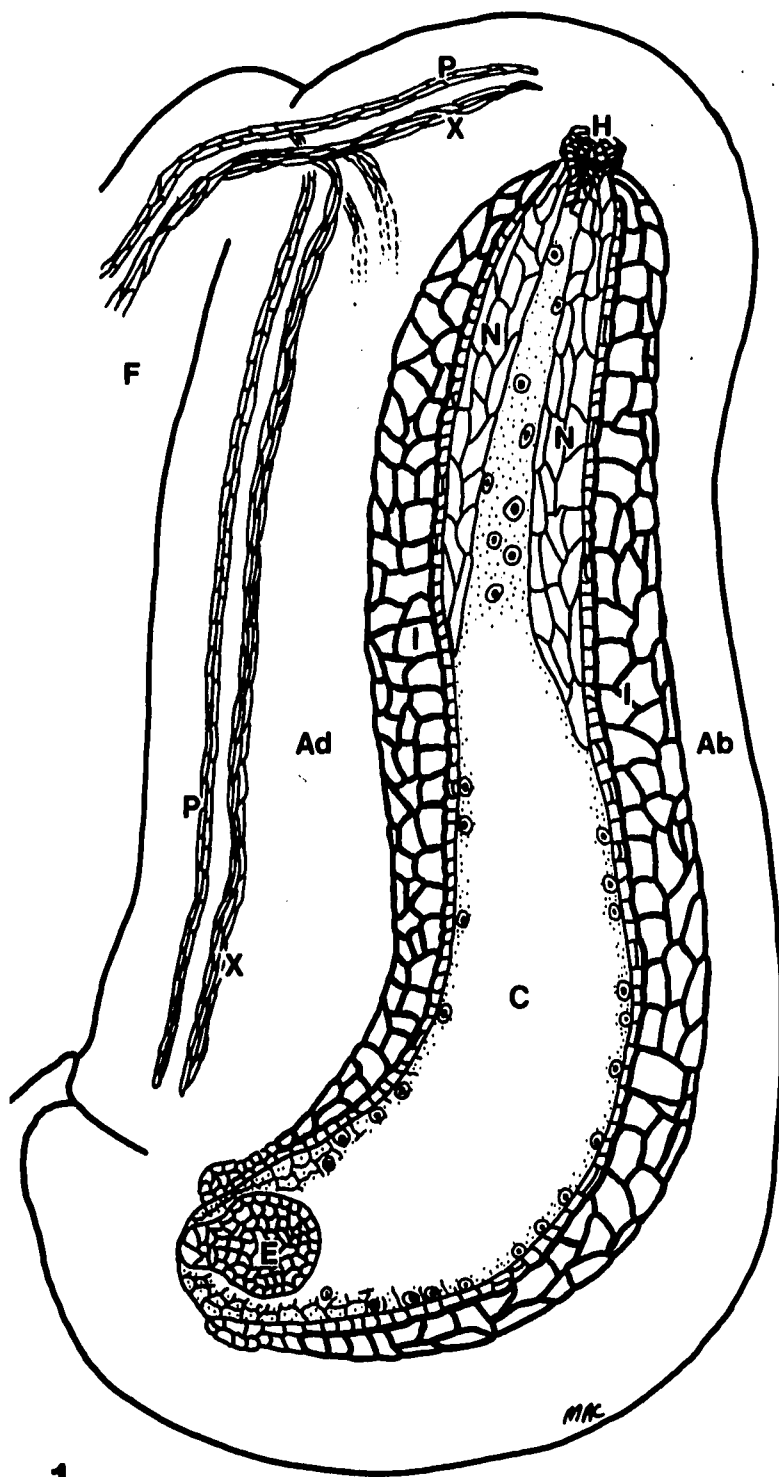
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APPENDIX

Figure abbreviations: Ab = abfunicular outer integument; Ad = adfunicular outer integument; C = central vacuole of embryo sac; CEs = central endosperm (cellular); Cp = chalazal process; E = embryo; En = endothelium; Ep = palisade epidermis; Es = endosperm; F = funiculus; H = hypostase; I = inner integument; N = nucellus; O = outer integument; P = phloem; PEs = peripheral endosperm; S = suspensor; W = wandlabrinthe; X = xylem; Z = zygote.

Fig. 1. Diagram of ovule in longitudinal section at globular embryo stage, showing arrangement of tissues and initial pattern of vascularization. Funicular vascular strand trifurcates after entering ovule forming a chalazal strand and two traces which lie parallel to each other within adfunicular outer integument. Bar = 100 μ m.



Figs. 2-6. Zygote stage. Fig. 2. Apex of zygote in longitudinal section showing absence of a cell wall (arrows) and concentration of amyloplasts and osmiophilic bodies (arrow heads). Note presence of large amyloplasts with aggregates of starch surrounding apex of zygote in central cell. Bar = 1 μ m. Fig. 3. Autoradiograph viewed with DIC optics of micropylar one-half of embryo sac. Label (black silver grains) is concentrated at base of embryo sac, in zygote, micropylar-most nucellar cells, and thin cytoplasm lining central cell. Large amyloplasts (arrow heads) are virtually unlabeled. Arrows mark cuticle layer separating nucellus from inner integument. Note absence of cuticle at extreme micropylar end of embryo sac. Bar = 20 μ m. Fig. 4. Autoradiograph viewed with DIC optics of chalazal one-half of embryo sac. Label is concentrated at hypostase, inner nucellus, and thin cytoplasm of central cell. Large amyloplasts (arrow heads) are unlabeled. Arrows indicate cuticle; note its absence lateral to hypostase. Bar = 20 μ m. Fig. 5. Wall with two short transfer cell-like wall projections with plasmodesmatal connections between nucellar cells. Bar = 0.5 μ m. Fig. 6. Chalazal end of ovule with vascular strand terminus proximal to hypostase. Bar = 20 μ m.

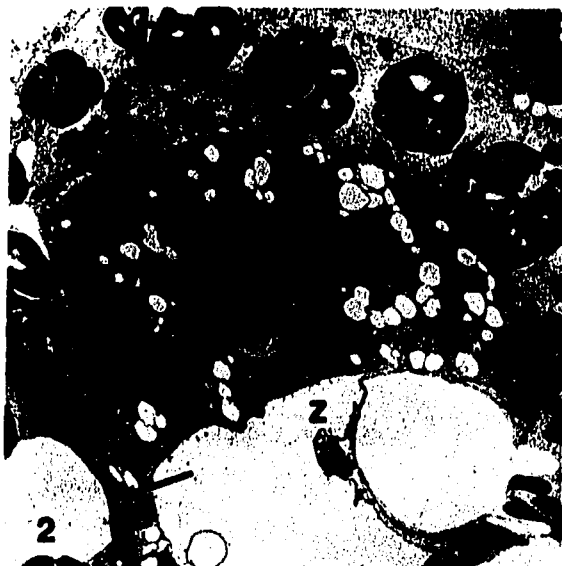


Fig. 7. Diagram of ovule in longitudinal section at zygote stage showing proposed pathway (arrow heads) and pattern of labeled ^{14}C in specific regions of ovule (number of silver grains per 1000 μm^2). Bar = 50 μm .

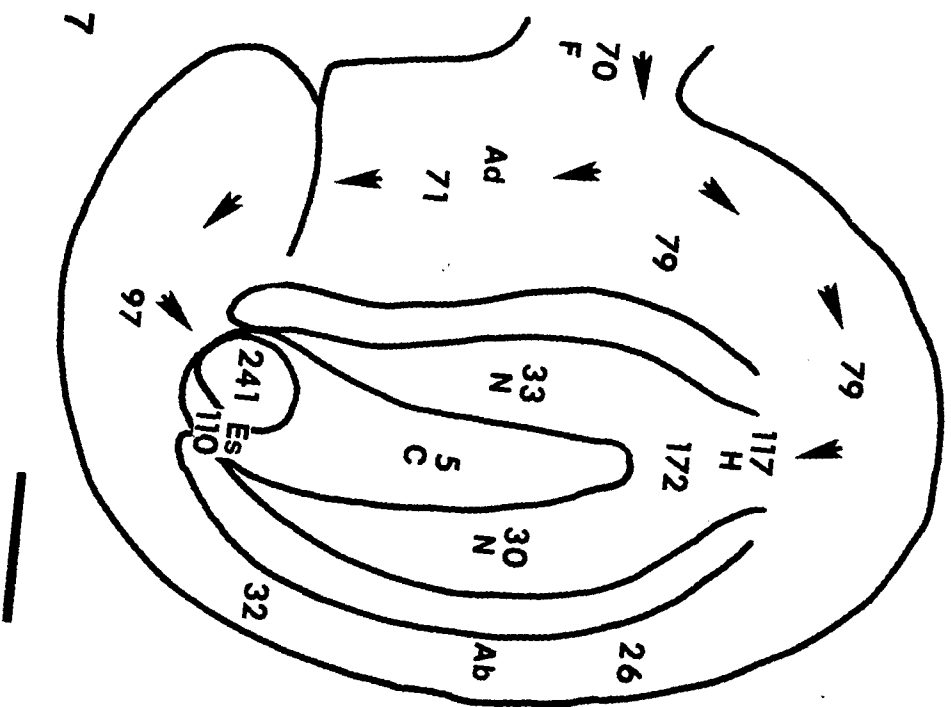
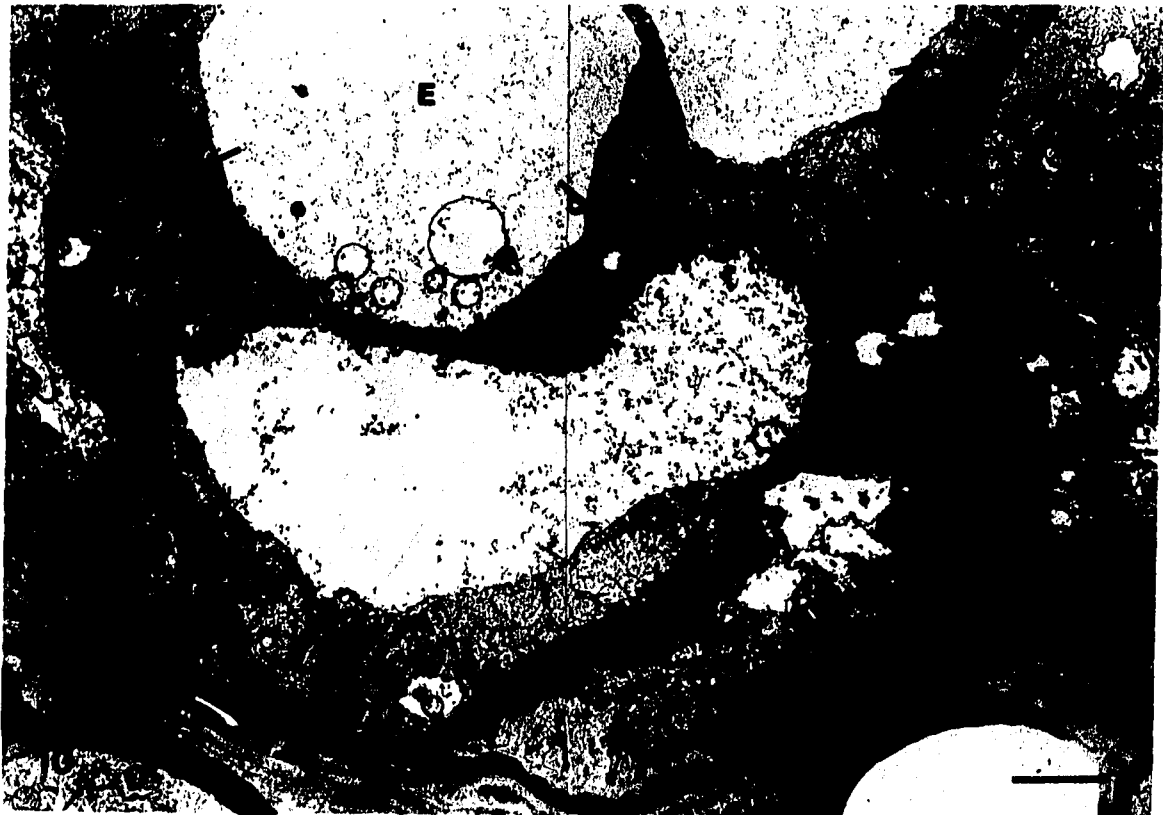
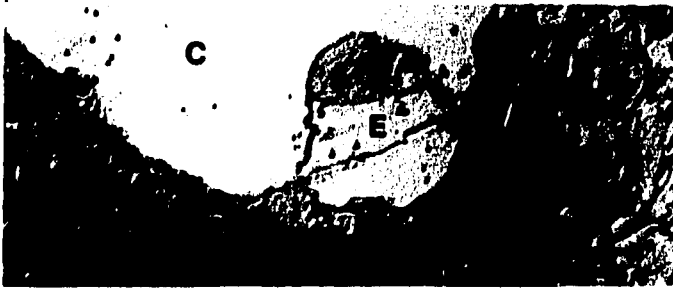
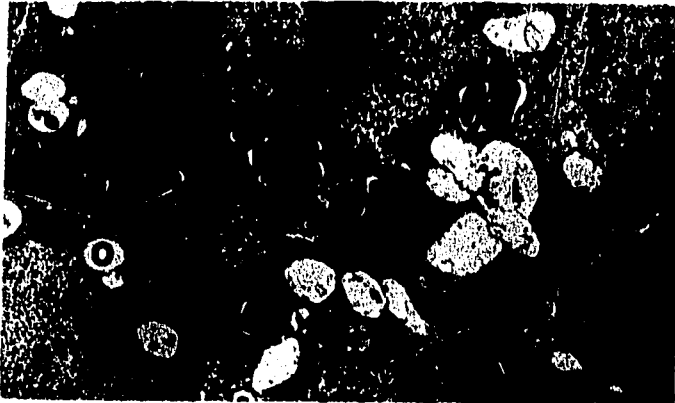


Fig. 8. Interface of inner and outer integuments at zygote stage. Note lack of plasmodesmatal connections between these two layers. Bar = 2 μ m. Figs. 9-11. Proembryo stage. Fig. 9. Young proembryo in oblique section showing abundant label. Note heavy concentration of label below proembryo in region of wandlabrinthe. Label also concentrated in thin endosperm cytoplasm lining embryo sac. Bar = 20 μ m. Fig. 10. Micropylar base of embryo sac with wall ingrowths of wandlabrinthe encircling base of proembryo. Note initial formation and position of transfer cell-like wall protuberances (arrows) in basal cells of proembryo. Bar = 2 μ m. Fig. 11. Accumulation of electron-dense material (arrows) at wall and within cytoplasm of a nucellar cell. Bar = 2 μ m.



Figs. 12-17. Globular embryo stage. Fig. 12. Autoradiograph viewed with DIC optics of pattern of labeling in micropylar integuments and embryo. Bar = 20 μ m. Fig. 13. Portion of embryo showing absence of a cuticle over its surface and surrounding cellular endosperm. Bar = 1 μ m. Fig. 14. Micropylar base of embryo sac showing position of wandlabrinthe (arrow). Basal suspensor cells have extensive wall ingrowths (asterisks). Bar = 2 μ m. Fig. 15. Autoradiograph viewed with DIC optics showing gradient pattern of labeling from adfunicular integuments to endosperm (undergoing cellularization) and absence of label in central vacuole. Bar = 20 μ m. Fig. 16. Detail of cuticle separating inner integument from endosperm near micropylar end of central cell. Note presence of thin areas in cuticle opposite peg-like wall ingrowths from central cell wall. Bar = 1 μ m. Fig. 17. Distinct gaps in cuticle of endothelium in micropylar one-half of embryo sac. Bar = 1 μ m.

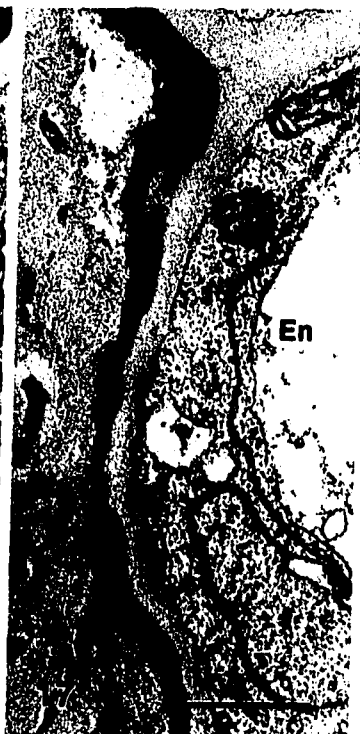
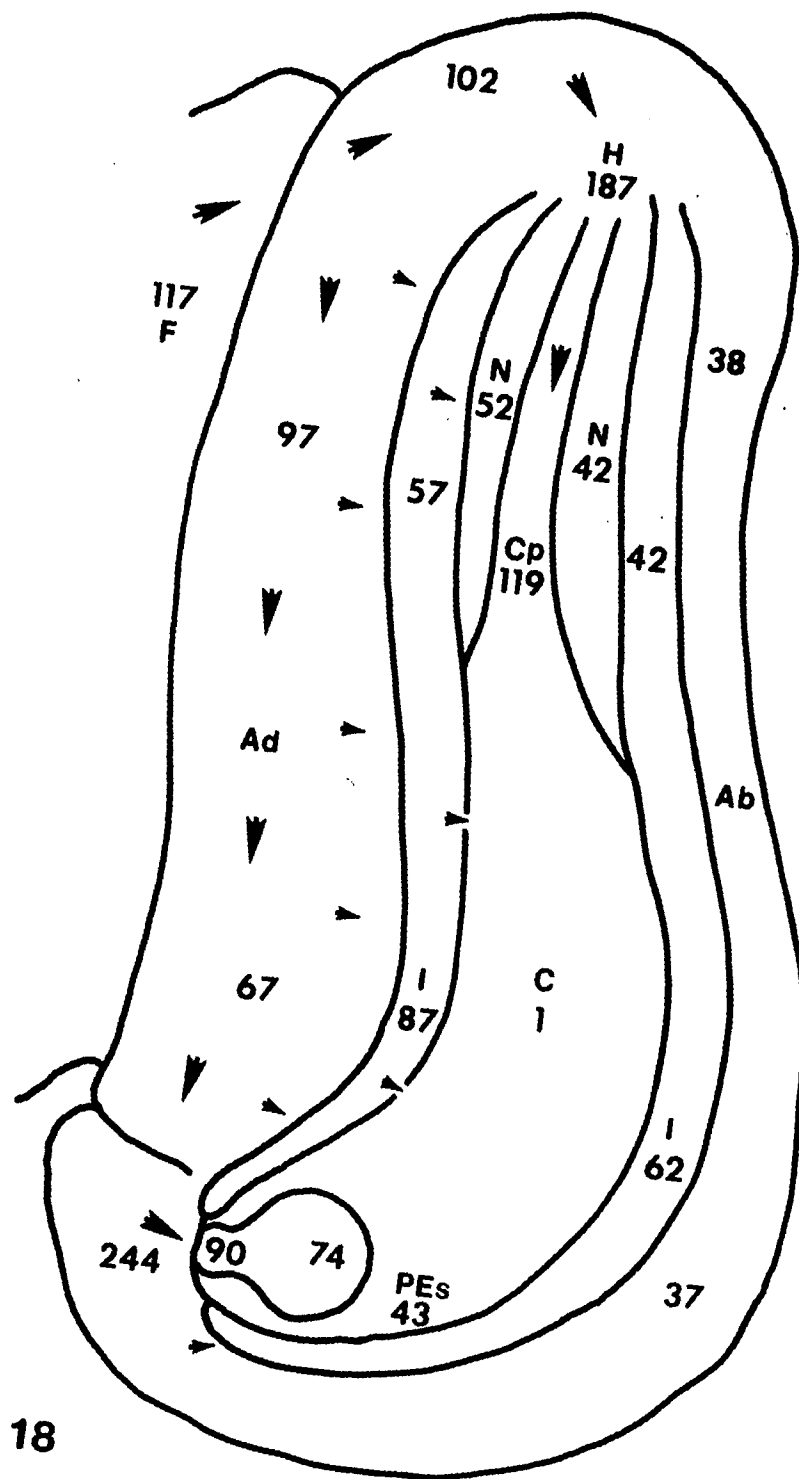


Fig. 18. Diagram of ovule in longitudinal section at globular embryo stage showing proposed major (large arrow heads) and minor (small arrow heads) pathways and pattern of labeled ^{14}C in specific regions of ovule (number of silver grains per $1000\ \mu\text{m}^2$). Bar = $100\ \mu\text{m}$.



Figs. 19-24. Globular embryo stage. Fig. 19. Cytological differences between outer (thick-walled) layers and inner layer (endothelium) of inner integument. Note intact cuticle separating endosperm from endothelial layer at chalazal one-half of central cell. Bar = 5 μ m. Fig. 20. Autoradiograph viewed with DIC optics showing accumulation of label in chalazal process which is filled with free-nuclear endosperm. Bar = 20 μ m. Fig. 21. Oblique longitudinal section, autoradiograph viewed with dark-field optics showing heavy concentration of label (white aggregates of silver grains) in funiculus and adfunicular outer integument. Note near absence of label in abfunicular outer integument. Bar = 100 μ m. Fig. 22. Position of chalazal vascular strand in relation to hypostase. Bar = 20 μ m. Fig. 23. Autoradiograph viewed with DIC optics showing heavy concentration of label (arrows) in outer integuments proximal to phloem of chalazal vascular strand. Bar = 20 μ m. Fig. 24. Outer integumentary cells just micropylar to base of embryo sac with high concentration of electron-dense material and starch. Bar = 5 μ m.

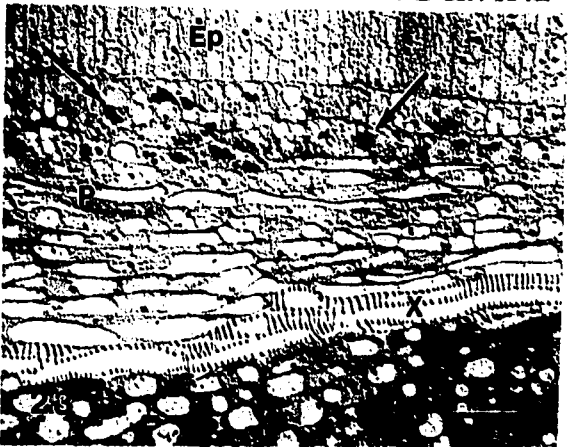
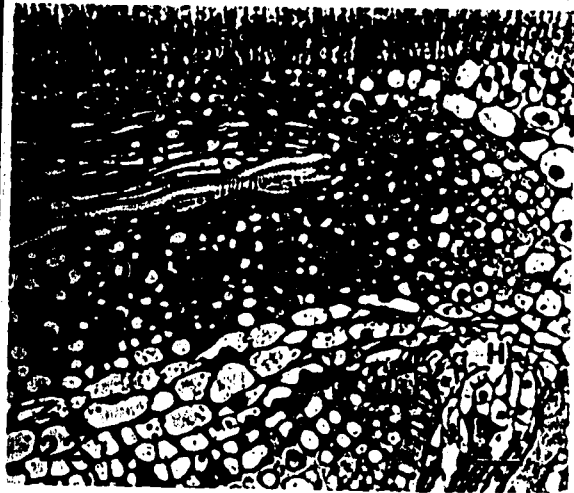
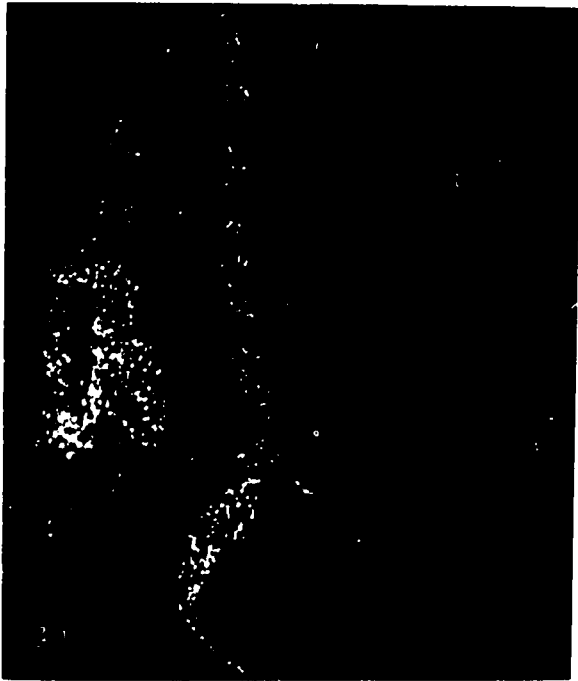
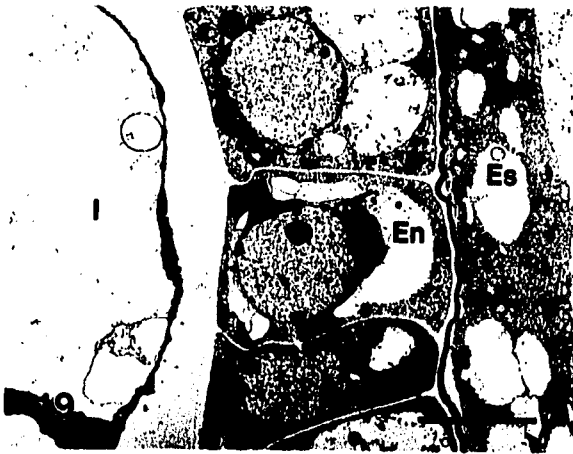


Fig. 25. Globular embryo stage. Extreme base of embryo sac and adjacent outer integuments. Note abundance and proximity of reserve-rich outer integumentary cells to embryo. Bar = 20 μ m. Figs. 26-29. Early heart-shaped embryo stage. Fig. 26. Embryo surrounded by highly vacuolate cellular endosperm. Bar = 100 μ m. Fig. 27.

Autoradiograph viewed with DIC optics of base of embryo sac and outer integument. Note pattern of label in reserve rich cells of outer integument, peripheral endosperm, and suspensor. Bar = 20 μ m. Fig. 28. Base of embryo sac showing suspensor and extent of wandlabrinthe (arrows). Bar = 20 μ m. Fig. 29. Longitudinal section through lateral outer integument showing anastamosing venation pattern and distinct sieve elements (arrow). Bar = 100 μ m.

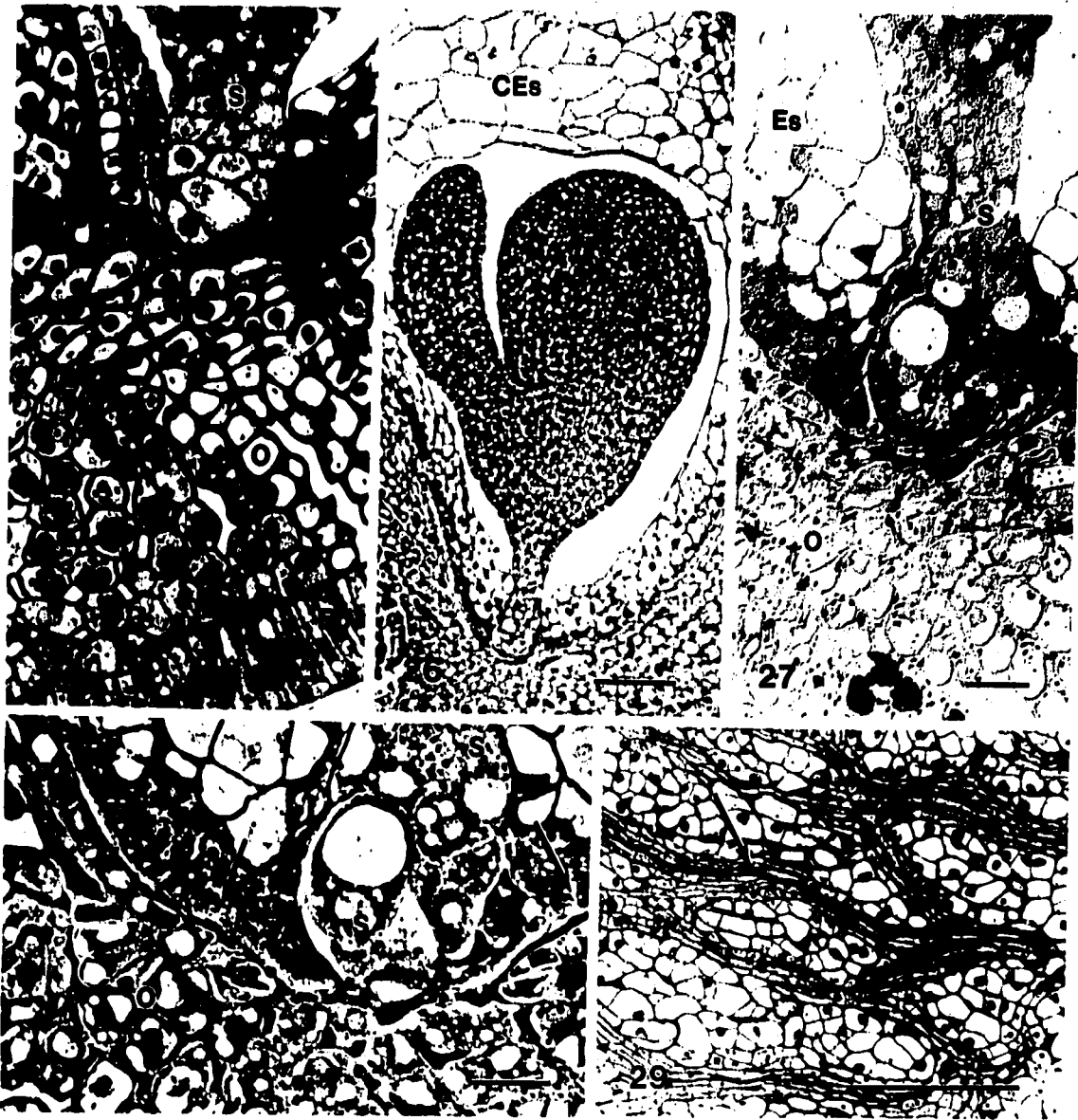
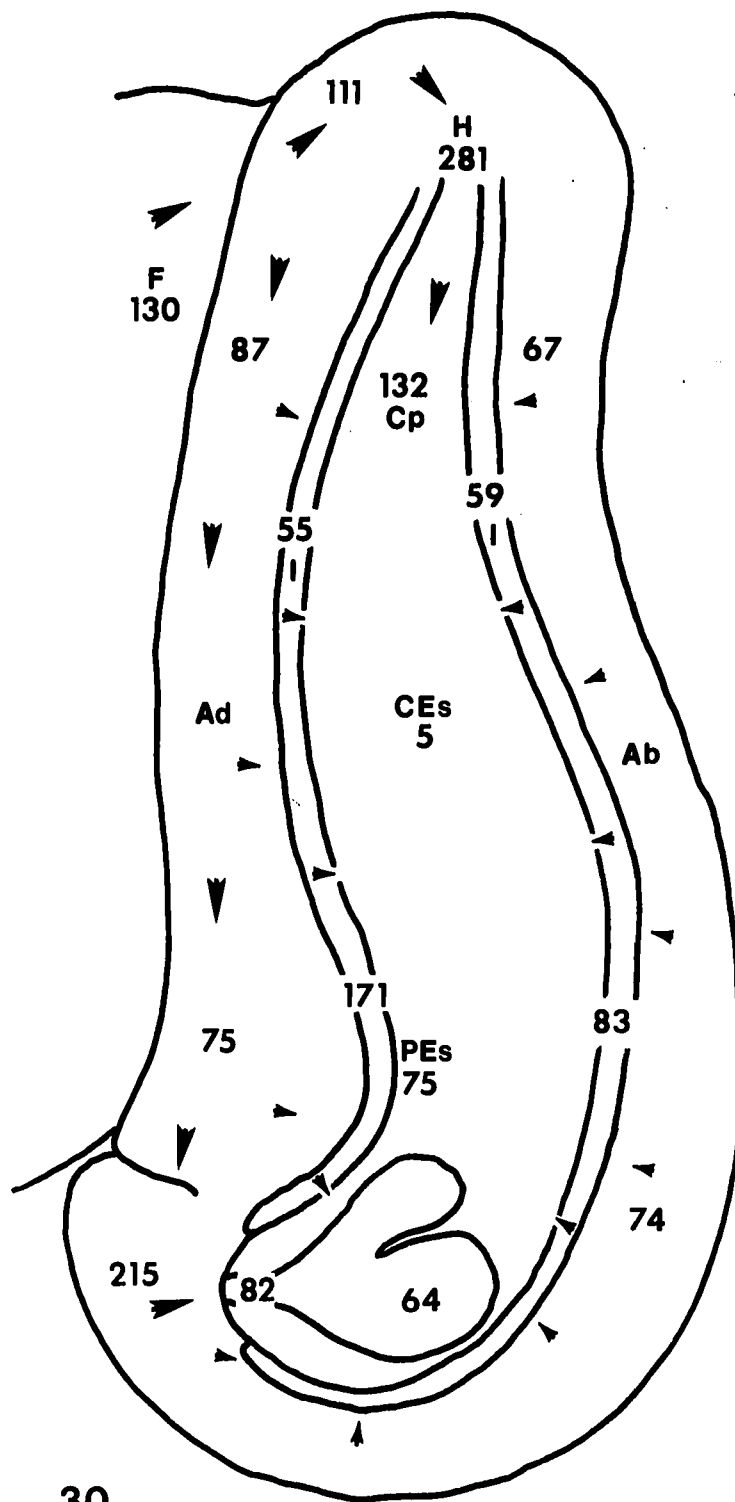
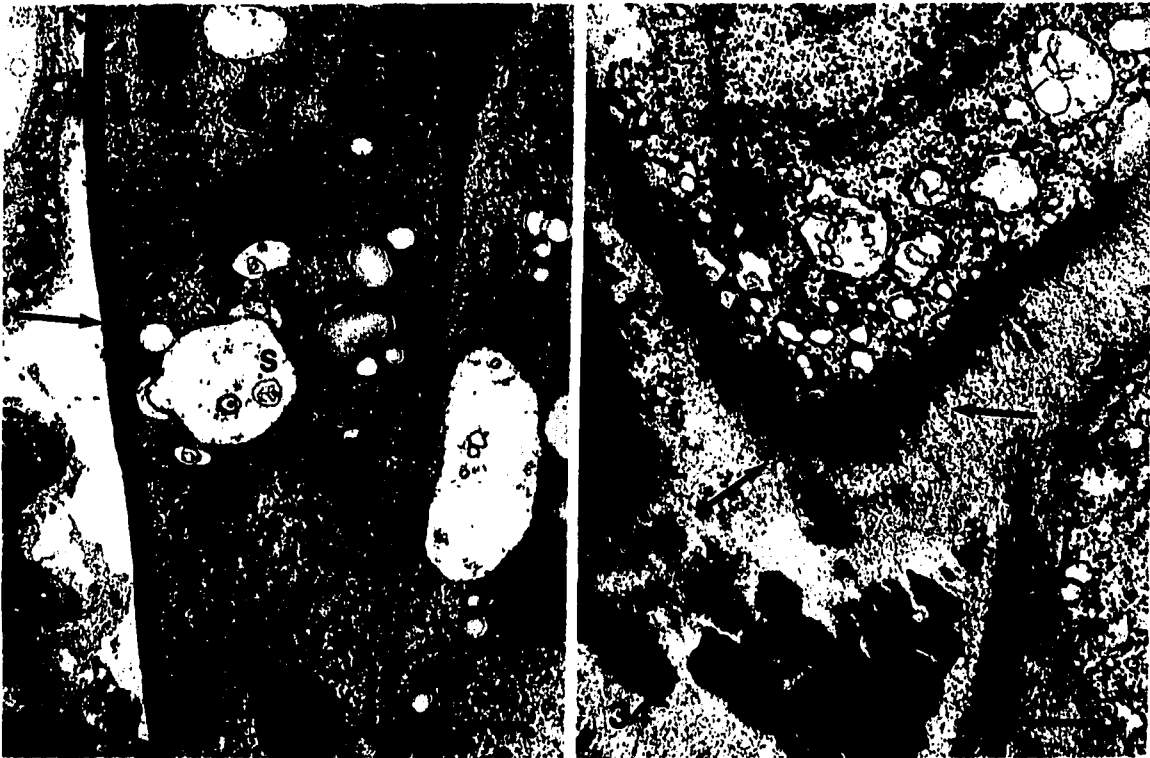
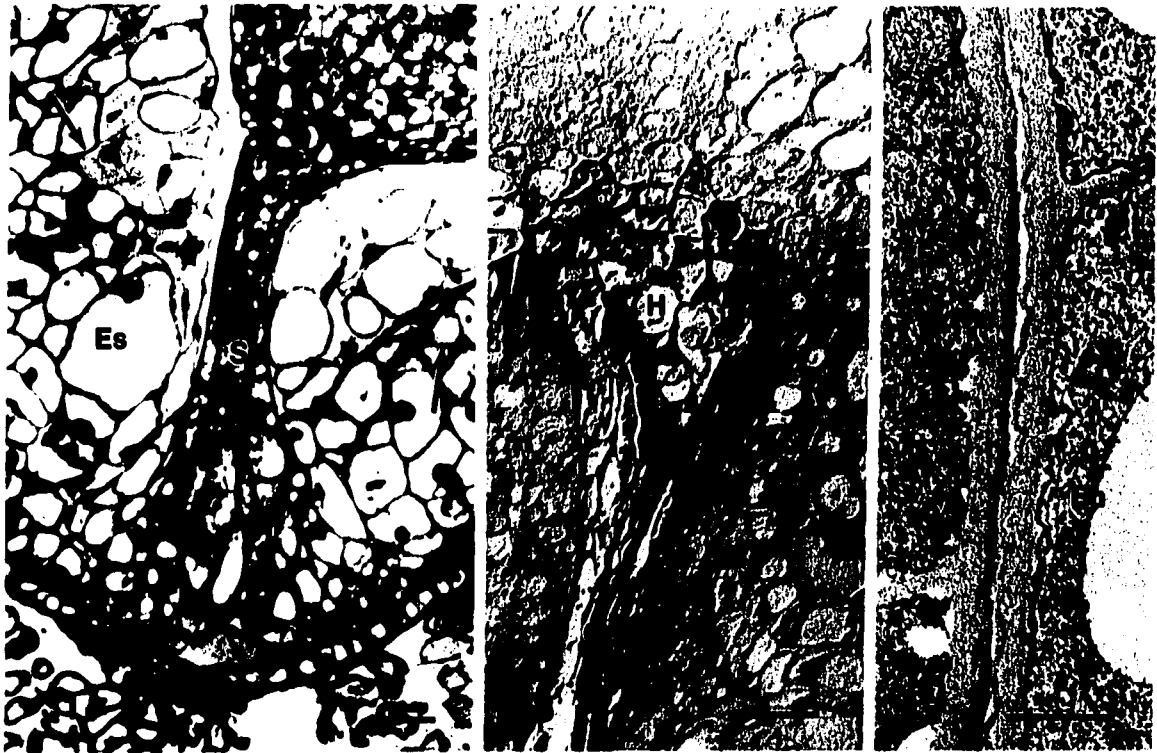


Fig. 30. Diagram of ovule in longitudinal section at early heart-shaped embryo stage showing proposed major (large arrow heads) and minor (small arrow heads) pathways and pattern of labeled ^{14}C in specific regions of ovule (number of silver grains per $1000\ \mu\text{m}^2$). Bar = $200\ \mu\text{m}$.



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Figs. 31-35. Late heart-shaped embryo stage. Fig. 31. Section of base of embryo and degenerating endosperm (arrows). Bar = 20 μ m. Fig. 32. Longitudinal section of suspensor cells showing abundance of amyloplasts. Note cuticle on surface of suspensor (arrow). Bar = 2 μ m. Fig. 33. Autoradiograph viewed with DIC optics of chalazal process (occluded by cellular endosperm) and hypostase in longitudinal section. Note abundance of label in and adjacent to process. Bar = 20 μ m. Fig. 34. Degenerating endosperm. Note loose fibrillar material at endosperm cell wall (arrows) associated vesicles within cytoplasm, and presence of electron-dense material (asterisk) between degenerating cells. Bar = 1 μ m. Fig. 35. Section at interface of endosperm and endothelium showing absence of cuticle. Bar = 1 μ m.



**PAPER 3: NUCLEAR SIZE AND DNA CONTENT OF THE EMBRYO AND
ENDOSPERM DURING THEIR INITIAL STAGES OF DEVELOPMENT IN
GLYCINE MAX (FABACEAE)**

**NUCLEAR SIZE AND DNA CONTENT OF THE EMBRYO AND ENDOSPERM
DURING THEIR INITIAL STAGES OF DEVELOPMENT IN GLYCINE MAX
(FABACEAE)**

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ABSTRACT

A technique was developed for isolating embryo sacs from ovules of soybean and for separating embryo from endosperm. Image analysis and cytophotometry were used to determine the relative mass of DNA and size of nuclei of endosperm and embryo cells. Analyses were done at the globular through late heart-shaped embryo stages to correlate ploidy level or nuclear size, and differentiation in these tissues. Mean size of embryo nuclei was fairly constant through all stages studied. Ploidy condition of the embryo was stable, 95%-99% of the nuclei were distributed in a bipolar pattern by relative mass at 2C and 4C. Few embryo nuclei (3%) had ploidy levels above 4C at the late heart-shaped embryo stage. Variability in size of endosperm nuclei seemed correlated with the morphological state of these nuclei (free-nuclear vs. cellular). Most endosperm cells did not show significant polyploidy with 84%-92% of nuclei in the expected 3C-6C range, but some nuclei with elevated ploidy levels were noted during endosperm cellularization. Endosperm senescence was correlated with nuclear DNA loss over time. Polyploidy seems to have no direct role in the early differentiation of the soybean embryo and endosperm, but these stable conditions may be necessary for the early establishment of the embryo.

INTRODUCTION

The endosperm is a unique tissue formed during double fertilization. As the endosperm develops, its nuclei may become polyploid and have maximum levels of 384C in maize (Tschermak-Woess and Enzenberg-Kunz, 1965), 96C in Phaseolus vulgaris (Nagl, 1970), 24C in Triticum aestivum (Chojacki, Bayliss, and Gale, 1986), 96C in Borassus flabellifer (Stephen, 1974) and 3072C in Echinocystis lobata (Turala, 1966). Not all nuclei within a given endosperm exhibit these high ploidy levels inasmuch as cytological differentiation within this tissue exists. The aleurone endosperm of Oryza sativa exhibited no polyploidization of its nuclei, retaining the base 3C-6C ploidy level, but greater than 20% of the central endosperm cells attained the 24C level (Ramachandran and Raghavan, 1989). A similar pattern existed in Zea mays, in which the central endosperm cells evidenced a mean DNA content up to 20-fold greater than that of peripheral endosperm cells (Kowles and Phillips, 1985, 1988). Numerous examples also have been reported in the differential polyploidization of the endosperm proper and the haustoria of various taxa (Chopra and Sachar, 1963; Turala, 1969).

The differentiation of the young angiosperm embryo into distinct organs may suggest a cytological differentiation of its tissues similar to that seen in the endosperm. The nuclei of the embryo proper of Phaseolus coccineus contained only two base levels of DNA (2C and 4C), whereas the nuclei of the suspensor proper and the large suspensor basal cells contained large polytene chromosomes with ploidy levels of 2C-64C and 256C-8192C, respectively (Brady, 1973). Other taxa have demonstrated cytological distinction between embryo proper and suspensor such as in Tropaeolum majus (Nagl, 1976), various species of Phaseolus (Nagl, 1974), and also, between cotyledons and embryo axes in Brassica napus (Silcock et al., 1990), Phaseolus vulgaris (Johnson and Sussex, 1990) and other

papilionaceous legumes (Bryans and Smith, 1985). These results suggest a division of labor of embryo and endosperm tissues that may be a result of the increased ploidy level of their cells. D'Amato (1952) suggested further that polyploidy in the embryo may directly affect differentiation and, therefore, function of these tissues. The gradient ploidy levels in the epidermal cells (2C), hypodermal cells (8C-32C), and central storage cells (64C) of the cotyledons of Pisum sativum (Scharpe and Van Parijs, 1973) seem to support this premise.

Therefore, polyploidy in endosperm and embryo tissues is likely to play an important functional role due to its prevalence in a number of diverse genera. In this study we report on the relative mass of DNA and the size of nuclei in soybean endosperm and embryo at the globular through the late heart-shaped embryo stages of development (6-35 d postfertilization, dpf), to determine whether polyploidy has any relevance to morphogenesis in these soybean tissues. The small size of the soybean ovules at these early stages and difficulties in obtaining a sufficient number of endosperm cells from this "nonendospermic" legume have challenged our abilities to study the endosperm and embryo tissues for ploidy changes. Because of these difficulties, we have developed a technique for the isolation of embryo sacs that allows us to obtain homogeneous samples of embryo and endosperm nuclei for cytophotometric analysis. In the present study, we utilize this new technique to analyze the embryo and endosperm nuclei in a normal system to serve as a model for understanding ploidy and morphogenesis in these tissues.

MATERIALS AND METHODS

Plants of Glycine max (L.) Merr. cv. Harosoy were germinated and grown in the USDA-ARS FCR glasshouse at Iowa State University, Ames. Ovules were dissected at various stages 6-35 dpf. Fertilization occurs approximately 24 hr after pollination in soybean. Ovules were fixed in fresh 10% formalin for 12 hr at room temperature. Ovules were washed in distilled water (2 hr), and the integuments superficially nicked. Ovules were placed in 5% cellulase (Cellulysin-Calbiochem, La Jolla, CA) plus 5% pectinase (Macerase-Calbiochem) in 5% mannitol solution for 3 hr at 37 C. They were washed in 5% mannitol (1 hr), and the integuments were dissected away to expose the embryo sacs (Figs. 1, 2). A modified Feulgen technique (Berlyn and Miksche, 1976) was used to stain the embryo and endosperm nuclei as follows: embryo sacs were hydrolyzed in 1 N HCl at 60 C for 10 min, and the hydrolysis reaction was stopped by a quick rinse in cold distilled water (4 C). The embryo sacs were placed in Schiff's reagent for 2 hr at 4 C in the dark, bleached in fresh 4% sodium meta-bisulfite for 30 min, and postwashed for 20 min in distilled water. Removal of the integuments allowed for consistent staining of the endosperm and embryo nuclei. No variability of staining was noted among ovules at the same stage of development. After staining, embryos were dissected from the endosperm tissue, and both tissues were squashed under coverslips on separate glass slides (Figs. 3, 4). The slides were frozen with liquid CO₂, and the coverslips removed. Slides were dehydrated in a graded ethanol series, then placed in xylene, and a coverslip was mounted with Permount. The nuclei were observed with a Leitz MPV microscope photometer fitted with a Leeds and Northrup 2430 DC galvanometer, and a transmitted illuminating system using a Leitz in-line mirror monochromator and a stabilized 150 W pressure xenon lamp. The two-wavelength method was used to determine the relative mass (RM) of the nuclear

DNA (Ornstein, 1952; Patau, 1952). As a standard, mature leaf tissues were digested, the nuclei stained, and the cells squashed, as described. Because cells of the leaf were nondividing, the nuclei of this tissue were used to determine relative DNA mass of a resting diploid nucleus. The 2C value (diploid amount of DNA) was equal to a relative mass of 125.0.

A Leitz microscope fitted with a video camera (COHU model 4815-5000) connected to a video digitizer (Colorado Video, Inc., Boulder, CO, model 270A) was used to digitize bright-field images to determine nuclear size of embryo and endosperm tissues. The digitized images were saved and manipulated with a Kevex Delta IV system and a Kevex Automated Image Analysis software program.

RESULTS

Embryo and endosperm morphology -- The embryo and endosperm passed through distinct morphological changes in the first 35 dpf. The globular embryo (glob) was formed 6–10 dpf, and was composed of an undifferentiated embryo proper and a short multiseriate suspensor. The bulk of the endosperm was free-nuclear at this stage of development, but cellularization of the endosperm began at the micropylar end of the embryo sac. The early heart-shaped embryo (early ht) was marked by the formation of the two cotyledon primordia at 11–16 dpf, and the cellular endosperm occupied greater than one-half the volume of the embryo sac.

At 17–25 dpf, the midheart-shaped embryo (mid ht) developed its polar meristems, and the cotyledonary cells began to fill with storage reserves. The endosperm was completely cellular at this stage, except for the chalazal process, which remained free-nuclear. These latter nuclei were generally hypertrophied in comparison with the nuclei of central and peripheral endosperm cells. The embryo enlarged rapidly to occupy approximately 75% of the embryo sac at 26–35 dpf, and its cotyledons continued to fill with storage reserves at the late heart-shaped embryo stage (late ht). At this stage of development, the embryo had encroached upon the central endosperm cells, and only the peripheral and the chalazal free-nuclei of the endosperm remained within the embryo sac.

Embryo nuclei -- The mean size (area) of the embryo nuclei varied little through all stages studied (Table 1). Embryo mean nuclear size at the late ht stage ($19.5 \mu\text{m}^2$) was slightly greater than at earlier stages which ranged from 14.7 – $16.1 \mu\text{m}^2$. Within each developmental stage, size of individual nuclei varied considerably. The largest nuclei were noted in the late ht stage ($64.5 \mu\text{m}^2$); the maximum sizes in the preceding stages were 35.4 –

47.8 μm^2 . A minimum nuclear size of 4.0–4.4 μm^2 was observed at each developmental stage, a size comparable to that of the smallest nuclei in the mature leaf tissue. In general, the extreme values noted for nuclear size made up less than 2% of the total nuclei in a given embryo. The mean nuclear size of the mature leaf tissue was 9.6 μm^2 , with minimum and maximum areas of 4.8 μm^2 and 13.9 μm^2 , respectively.

The embryo ploidy levels (Table 2) were stable from the glob stage to the mid ht stage (2.3C, 2.4C), with only a slight increase in mean nuclear DNA relative mass to 167.1 (2.7C) at the late ht stage. In all stages studied, 95%–99% of all nuclei were in the expected 2C–4C range. Significant amplification of DNA above the 4C level (Table 3) was noted only at the late ht stage, at which 3% of the nuclei had a maximum of 5.7C. Nuclear DNA mass levels lower than 2C were noted at each stage of development, but only in a small number of nuclei (Table 3). The embryo nuclei were distributed in a bipolar pattern by RM in all stages reported (Fig. 5). The peak distribution of nuclei at 125.0 RM was equal to 2C, as determined from nondividing nuclei of mature leaf tissue. The 4C level was represented by the second distribution peak of nuclei at approximately 250.0 RM. A curious population of embryo nuclei at approximately 180.0 RM was intermediate in ploidy between 2C and 4C at the late ht stage (Fig. 5). This intermediate peak (probably nuclei in S-phase) indicated the high level of mitotic activity observed at this stage of development.

Endosperm nuclei -- The mean size (area) of the endosperm nuclei varied considerably among the different stages of development (Table 1). At the glob stage, the embryo sac was filled predominantly with large free-nuclei although some cellular endosperm was observed at the micropylar end. These endosperm nuclei had a mean size of 37.1 μm^2 and reached a maximum size of 164.0 μm^2 . The endosperm nuclei at the early ht and mid ht stages were significantly smaller than those at the glob stage (Table 1). The endosperm at

the early ht stage was a heterogeneous tissue consisting of free-nuclei and cellular endosperm with a mean nuclear size of $20.8 \mu\text{m}^2$. Cellularization of the endosperm was nearly complete at the mid ht stage, and these nuclei had a mean nuclear size of $25.3 \mu\text{m}^2$. A large portion of the endosperm degenerated as the embryo expanded into the embryo sac during the late ht stage. The mean size of the nuclei at this developmental stage was $34.1 \mu\text{m}^2$, and very large nuclei with a maximum of $195.0 \mu\text{m}^2$ were also observed. At all stages, the endosperm nuclei were significantly larger than their counterparts in the embryo (Table 1).

The ploidy level of the endosperm nuclei was fairly stable at all stages (Table 2), with 84%-92% of all nuclei in the expected 3C-6C range. The mean DNA RM for endosperm nuclei at all stages ranged from 206.2 RM to 237.3 RM (3.3C-3.8C). Only a few endosperm nuclei at the early ht (7.1%) and mid ht (2.4%) stages showed increased ploidy, greater than 6C (Table 3), and the maximum C-value was 9.7. The mean RMs of the endosperm nuclei were statistically (t-test) greater than those of the embryo (Table 2).

Surprisingly, the endosperm nuclei did not show a bipolar RM distribution at 3C and 6C (Fig. 5). In all stages a distinct peak representing the 3C condition was seen at 190-200 RM, but the expected 6C peak at 380.0 RM was conspicuously absent at each stage in the endosperm. Instead, the peak distribution of endosperm nuclei at the glob, early ht, and mid ht stages had a smaller than expected nuclear DNA RM at 350.0 (5.5C). This trend continued into the late ht stage, during which there was further reduction in the nuclear DNA RM of this tissue. A broad distribution peak at 280-325 (4.4C-5.1C) was observed at this stage. An increasing number of endosperm nuclei exhibited DNA levels below 3C as well, during the development of the endosperm (Table 3). At the glob stage, 7.9% of the endosperm nuclei had C-values below the expected ploidy level, and this trend continued through the late ht stage (12.8%).

DISCUSSION

Previous studies have found a direct correlation between the incidence of polyploidy and increased nuclear volume in endosperm and embryo tissues (D'Amato, 1984; Bryans and Smith, 1985; Silcock et al., 1990). Because polyploid nuclei were rare in the present study, no direct correlation could be made between nuclear size and DNA content. Nevertheless, it can be stated that an increase in the endosperm mean nuclear size did not reflect elevated ploidy levels. The endosperm mean nuclear size showed distinct variation at each stage of development (Table 1), but the mean RM of these nuclei (Table 2) did not reflect this variation in size.

The variation in size of the endosperm nuclei likely reflects the morphological status of this tissue, and not the DNA content of its nuclei. The large value for the mean nuclear size observed at the glob stage reflects a predominance of acellular endosperm with its free-nuclei. The mean size of the endosperm nuclei decreased as cellularization of the endosperm proceeded, and the latter was nearly completed between the early ht and mid ht stages. It is unclear why individual nuclei are reduced in size during cellularization of the endosperm. In previous work (unpublished data), we noted that the bulk of the endosperm cells rarely divided after cellularization. In the absence of mitosis, it is probable that much of the biosynthetic machinery necessary for division may be lost and, as a consequence, the nuclei become more compact.

Most of the central endosperm is eventually lost due to the encroachment of the embryo into the embryo sac during the late ht stage. Only the large free-nuclei of the chalazal process and the rapidly dividing peripheral layer remained. Therefore, the increase in mean nuclear size of the endosperm observed at this stage (Table 1) likely reflects a

disproportionate reduction in the number of small nuclei of the central endosperm, and not a general increase in nuclear size for the endosperm.

The embryo passed through a number of distinct morphological changes in the early stages of its development, but this was not reflected cytologically. The RM distribution of embryo nuclei at 2C (G1-phase) and 4C (G2-phase), and the reduced distribution at the intermediate S-phase, suggests a normal pattern for mitotically dividing diploid tissues (Fig. 5). The ploidy level of these tissues was stable, with a large percentage of the embryo nuclei in the 2C-4C range (Table 3). Only at the late ht stage was there any indication of increased ploidy, with a small number of nuclei (3%) above the expected 4C value (Table 3). This increase coincided with a slight elevation in mean ploidy (2.7C); (Table 2). These latter results are similar to those noted in G. max cv. Dare (Dhillon and Miksche, 1983). They observed 5%-7% of the cotyledon nuclei above the 4C level and a mean DNA content of 2.7C at 15 days postanthesis through the "very early maturation" stage. The mean DNA content and the number of polyploid nuclei increased as the cotyledons continued to mature. These researchers found a direct correlation between DNA content in the cotyledon nuclei and increased RNA and protein synthesis in these cells. In the present study, the cotyledons began to accumulate storage reserves at the mid ht stage, and this continued through the late ht stage. It is possible that the presence of embryo nuclei with DNA content greater than 4C marks the initiation of protein synthesis and its accumulation in the cotyledons that is similar to that found in G. max cv. Dare. The incidence of polyploidy and its relation to protein accumulation were also observed in the cotyledonary cells of Pisum sativum by Scharpe and Van Parijs (1973).

As a result of the variation in mean nuclear DNA content of the endosperm nuclei (Table 2), no distinct correlation could be made between ploidy level and endosperm differentiation. Although mean nuclear RM was fairly constant over time, the extreme

examples of nuclei with C-values greater than 6C (9.7C instead of expected 12C; possibly due to selective DNA amplification) and less than 3C may be indicative of some morphological or physiological change in this tissue. Cellularization of the bulk of the endosperm seemed to coincide with the presence of nuclei with increased DNA content at the early ht (7.1%) and mid ht (2.4%) stages (Table 3). These results and the near absence of such endosperm nuclei (above 6C) at the glob stage (initial stage of cellularization) and at the late ht stage (completed cellularization) suggest that a small population of endosperm cells may have a functional role in cellularization.

An increasing number of endosperm nuclei exhibited some loss of DNA content below the expected 3C level during development (Table 3). This loss was reflected in the RM frequency distribution of endosperm nuclei (Fig. 5). A loss of DNA was indicated by a shift in the RM of the endosperm nuclei in the G2 phase of DNA synthesis (375 RM or 6C) towards lower levels of DNA content (Fig. 5). After cellularization, the central endosperm cells seemed to degenerate with a loss of cytoplasm and organelles. Senescence of these cells likely included the loss of nuclear DNA and, therefore, an increased incidence of nuclei with reduced ploidy levels. A similar correlation between cellular degradation and DNA loss has been noted in the endosperm of maize (Kowles, Srienc, and Phillips, 1990) and wheat (Chojecki, Bayliss, and Gale, 1986) and in the senescent cotyledons of soybean (Chang, Miksche, and Dhillon, 1985).

Another possible explanation for the decline in DNA content of the endosperm nuclei is nutritive. Supplying nutrients necessary for growth of the embryo is a typical function of the endosperm of angiosperms. The loss of DNA may form a pool of free bases possibly serving as a ready source of deoxynucleosides and phosphates for the developing embryo (Dhillon and Miksche, 1983). Further research is necessary to determine the exact nature and consequence of DNA loss in this tissue.

In conclusion, the stable ploidy condition predominated in the early stages (6–35 dpf) of embryo and endosperm development in soybean. Thus, polyploidy had no direct role in the early differentiation of these tissues. The limited number of nuclei with elevated ploidy levels observed in this study was likely a consequence of differentiation, and not its cause. The absence of polyploidy in the early stages of zygote/embryo and endosperm development may be a necessary condition for establishment and success of the angiosperm embryo. Variability in ploidy of one or both of these tissues may result in a lack of embryo/endosperm compatibility and subsequent embryo abortion, as suggested by other researchers (White and Williams, 1976; Williams and White, 1976). These former conditions seem to be fulfilled in normal soybean, as evidenced in this study by the stable ploidy levels and the near constant ratio of DNA content between embryo and endosperm (2C:3C). Once the embryo is established, polyploidization of embryo and endosperm tissues may be a necessary requirement for the continued development of the embryo. This latter statement is a possibility, due to the prevalence of polyploidy in endosperm and embryo tissues of a number of diverse genera. The majority of studies observing polyploidy in these tissues have concentrated on stages of development during which the embryo is well established or nearly mature. Further research seems warranted, in these genera, to determine whether there is a continuum of polyploidy in the early stages of zygote/embryo and endosperm development as well. The technique presented here, for the isolation of embryo sacs, gives us the tool for analyzing these tissues at early stages of development.

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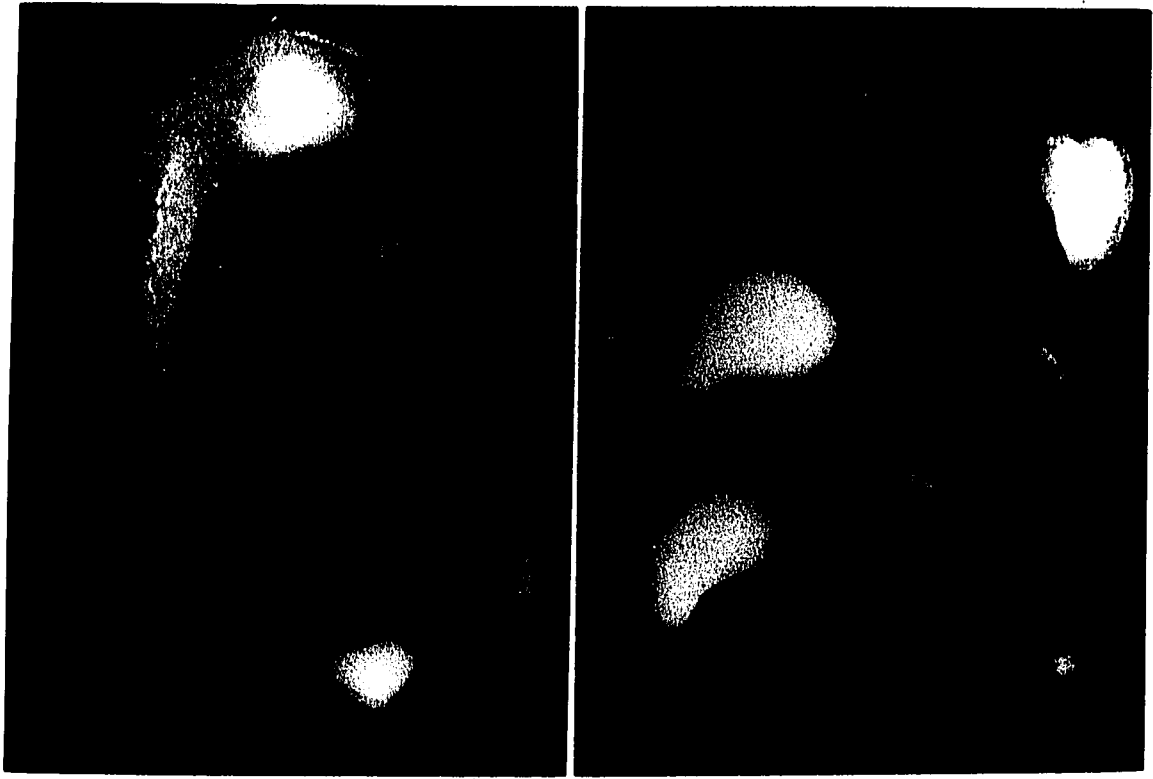
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APPENDIX

Figs. 1, 2. Photomicrographs of immature Glycine max ovules. Bars = 100 μ m. 1. Early heart-shaped embryo stage, ovule at left with integuments intact and embryo sac at right with integuments removed by enzymatic digestion. 2. Midheart-shaped embryo stage embryo sacs with integuments removed by enzymatic digestion.



Figs. 3, 4. Light micrographs of Glycine max embryo and endosperm squashes at midheart-shaped embryo stage; nuclei stained in Feulgen reaction. Bars = 20 μ m. 3. Population of embryo cells. 4. Population of endosperm cells.

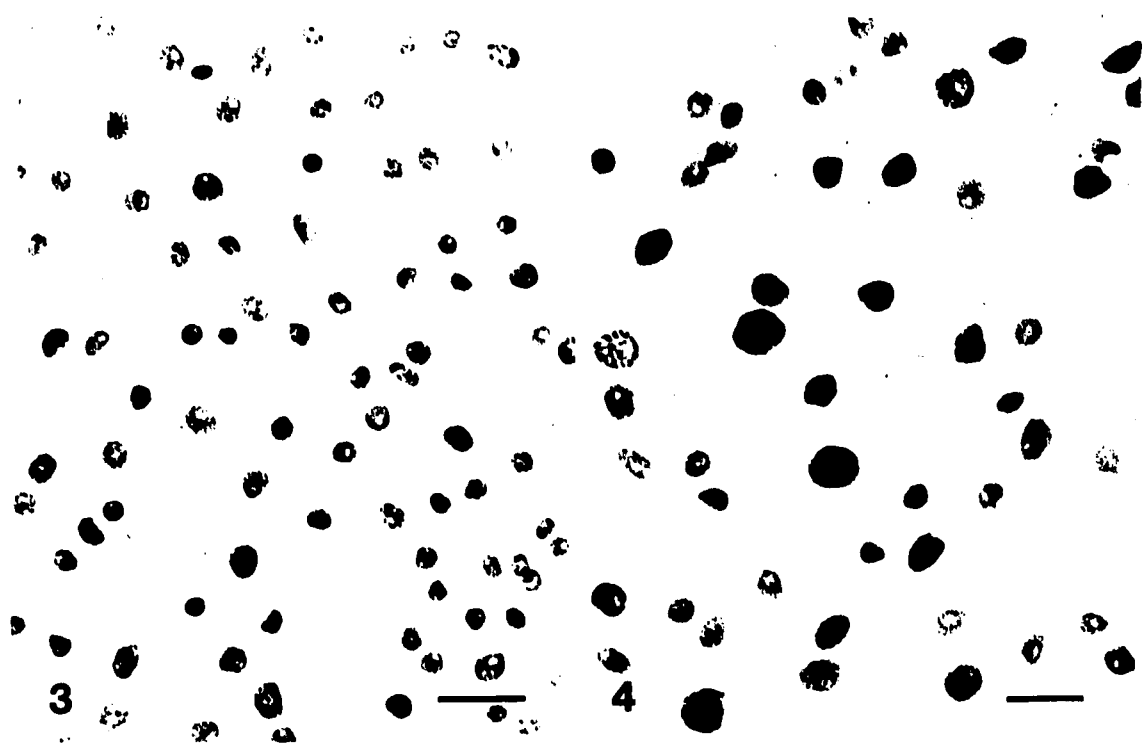


Fig. 5. Frequency distribution of embryo and endosperm nuclei by DNA relative mass (RM) at four stages of embryo development in Glycine max: GLOB = globular; EARLY HT = early heart-shaped; MID HT = midheart-shaped and LATE HT = late heart-shaped.

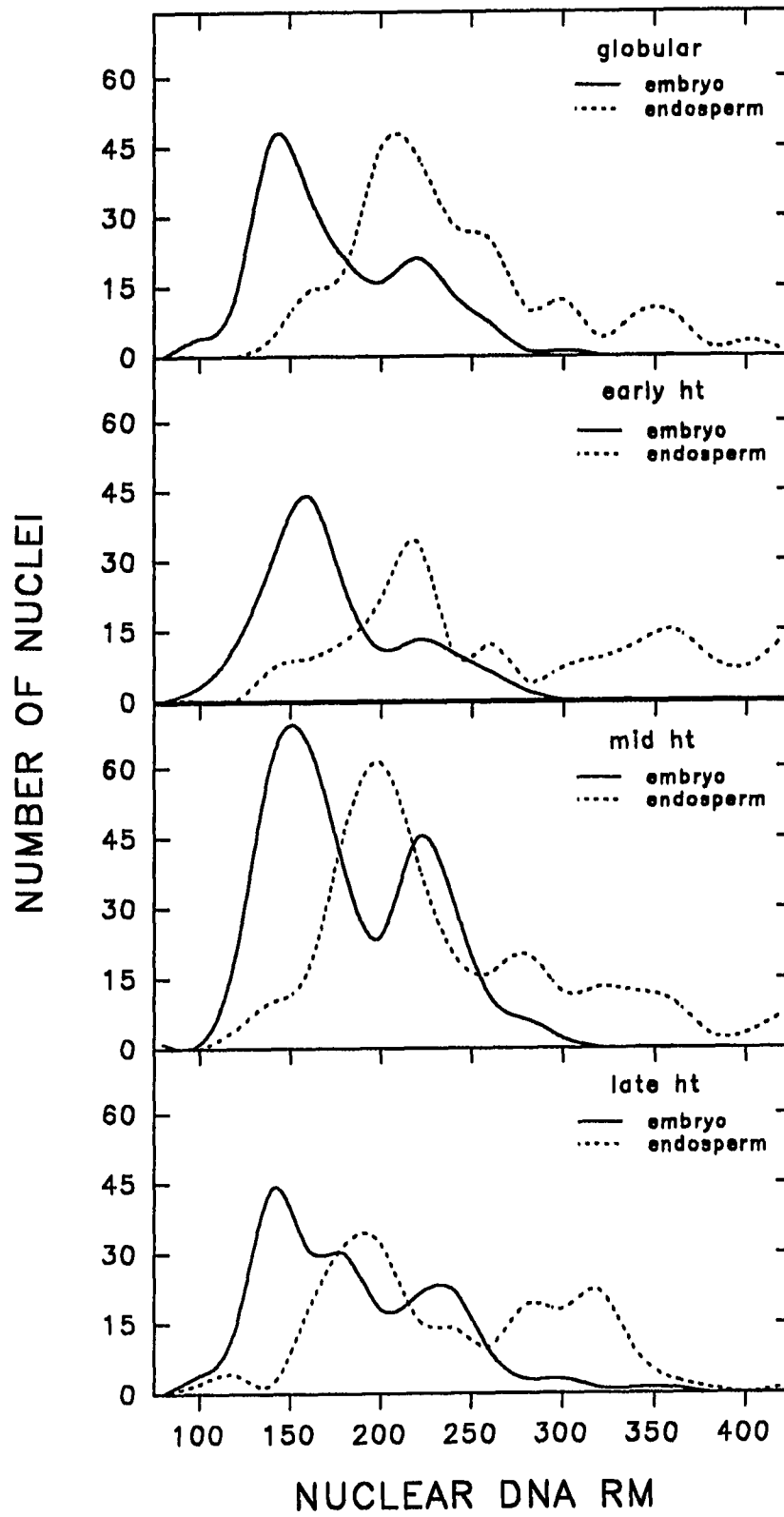


TABLE 1. Mean size (area in μm^2) of embryo and endosperm nuclei at various stages of embryo development^a

Stage	Embryo nuclei				Endosperm nuclei			
	Ovules	<u>N</u>	Mean	SD	Ovules	<u>N</u>	Mean	SD
Globular	5	330	16.1a	6.8	7	265	37.1c	25.8
Early heart	4	364	14.7a	5.5	4	248	20.8d	7.9
Midheart	6	1047	15.7a	7.1	6	845	25.3e	14.4
Late heart	4	1035	19.5b	9.8	4	873	34.1f	15.8

^aValues of means followed by the same lowercase letter are not significantly different at the $P \leq 0.01$ level, according to t-test.

TABLE 2. Mean DNA relative mass and C-value of embryo and endosperm nuclei at various stages of embryo development^a

Stage	Embryo nuclei					Endosperm nuclei				
	Ovules	<u>N</u>	Mean	SD	C-value	Ovules	<u>N</u>	Mean	SD	C-value
Globular	5	179	145.0a	39.3	2.3C	7	228	213.1c	49.7	3.4C
Early heart	4	155	142.5a	46.5	2.3C	4	183	237.3c	89.5	3.8C
Midheart	6	306	152.9a	42.7	2.4C	6	296	206.2d	75.0	3.3C
Late heart	4	202	167.1b	50.6	2.7C	4	203	223.5c	64.8	3.6C

^aValues of means followed by the same lowercase letter are not significantly different at the $P \leq 0.01$ level, according to t-test.

TABLE 3. Incidence (%) of embryo and endosperm nuclei with greater than or less than normal ploidy levels

Stage	N	Embryo		N	Endosperm	
		< 2C	> 4C		< 3C	> 6C
Globular	179	2.2%	0.0%	228	7.9%	0.0%
Early heart	155	1.9%	0.0%	183	8.7%	7.1%
Midheart	306	0.7%	0.7%	296	10.5%	2.4%
Late heart	202	2.0%	3.0%	203	12.8%	0.0%

GENERAL SUMMARY

Angiosperm ovule development provides a system to study the interaction of three genetically distinct sets of tissues toward the reproductive success of the seed. The sets of tissues are: 1) the maternal ovular tissues, consisting of the integuments, nucellus, and funiculus; 2) the endosperm; and 3) the embryo. Little is known concerning their interactions during early ovular, endosperm, and embryo development. A study of the pattern of assimilate movement in the ovule would give some insight into the interactive importance of these distinct tissues to the success of the embryo.

After fertilization the soybean embryo, endosperm, and ovule generally pass through a period of rapid growth and expansion in which their nutritional requirements are high. Within the ovular tissues, the embryo acts as a significant sink for translocated assimilates. The maturation of the embryo depends upon the successful flow of nutrients through the surrounding genetically distinct tissues.

The flow of labeled assimilates within the soybean ovular tissues followed a characteristic pattern. This pattern was strongly correlated with the differentiation of the tissues of the ovule and embryo sac over time. In spite of the fact that these tissues are genetically distinct I found that their development was highly interdependent. This integrated development seemed to be essential to the flux of nutrients toward the embryo sac and embryo.

A prime example of the integrated development of the tissues of the ovule and their effect on nutrient flux was observed during the globular embryo stage. At the zygote and proembryo stages of development label entered the embryo sac only through its micropylar and chalazal ends. But during the globular embryo stage there was a change in the pattern of label incorporation. Label continued to enter the embryo sac through its poles, but was

now supplemented by the movement of label into the lateral regions of the embryo sac via the inner integuments. This lateral movement was correlated with the coincident differentiation of the endothelial layer and fragmentation of its cuticle, the cellularization of the endosperm, and the vascularization of the adjacent outer integument. The resultant increase in the movement of nutrients into the embryo sac seemed to be timely, occurring shortly after the depletion of starch reserves within the central cell.

Any anomaly in the development of the ovular tissues, endosperm, or embryo could disrupt the nutrient flow within the ovule and ultimately lead to reduced embryo vigor or to abortion. Such may be the case in interspecific crosses of Trifolium (Williams 1987). In this study there was a strong correlation between the absence or abnormal development of the endospermic haustorium and ovule abortion.

Ovule abortion resulting from endosperm failure seems to be a common occurrence in interspecific crosses (White and Williams 1976; Arisumi 1982; Lin 1984). The Endosperm Balance Number (EBN) hypothesis was proposed by Johnston et al. (1980) to explain abnormal endosperm development in interspecific crosses. The EBN hypothesis suggests that there are factors (chromosomal loci) in the polar nuclei and sperm nucleus that must be in a balanced ratio for their compatibility and ultimate fusion to form a viable endosperm. An absence of compatibility between these nuclei results in a lack of fusion or in the abnormal development of the endosperm.

These compatibility factors are likely inherited by the embryo as well, but there are only two sets (sperm and egg) versus the three sets for the endosperm. Extrapolating from the EBN hypothesis, it may be suggested that EBN factors influence the closely related development of the endosperm and embryo, similar to that of the polar nuclei and sperm. If a balanced ratio of EBN loci between the embryo and endosperm is important, then stable ploidy levels also are requisite to sustain compatibility between these tissues. In the

present combined studies this was found to be true. No significant polyploidy was observed in the nuclei of the embryo and endosperm during their early stages of development (6-35 days postfertilization) and a constant ratio of DNA was observed 2C:3C, respectively. The results of this research suggest that these stable conditions are essential to the establishment of the embryo and endosperm. Any variation in the balanced ratio of DNA between these tissues may lead to incompatibility and destabilization of their interdependent development that may result in their failure and ovule abortion. The results of this research are in conflict with those of other genera. But the majority of studies that have observed polyploidy in these tissues utilized stages of development when the embryo and endosperm were well established or mature.

In conclusion, the development of the soybean embryo, endosperm, embryo sac, and other ovular tissues occurs in a series of interdependent stages that follow a highly orchestrated sequence of events through the first 35 days postfertilization. The spatial and temporal movement of labeled assimilates is well correlated with the combined differentiation of these tissues. The ploidy conditions of the embryo and endosperm do not reflect morphogenesis in these tissues, but do emulate the stable and interrelated development of the normal soybean embryo and endosperm.

Although, the problem of incompatibility resulting from interspecific crosses was not specifically addressed in this dissertation, the information and techniques utilized can now serve as a model to help elucidate the issue. Further studies should be conducted into the ploidy condition of the embryo and endosperm at their initial stages of development. If any incompatibility occurs, it is likely to be expressed at these early stages. Labeled $^{14}\text{CO}_2$ coupled with autoradiography also would be a useful tool in looking for abnormal patterns of labeled photosynthate flux in the ovules of incompatible crosses.

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